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(21) International Application Number: PCT/US96/02064 (22) International Filing Date: 15 February 1996 (15.02.96) (30) Priority Data: 08/389,926 16 February 1995 (16.02.95) US (71) Applicant: THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY [US/US]; Office of Technology Licensing, Suite 350, 900 Welch Road, Palo Alto, CA 94304-1850 (US). (72) Inventor: DZAU, Victor, J.; 12101 Dawn Lane, Los Altos Hills, CA 94022 (US). (74) Agent: HUGHES, A., Blair; Banner & Allegretti, Ltd., Ten South Wacker Drive, Chicago, IL 60606 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: INHIBITION OF PROLIFERATION OF VASCULAR SMOOTH MUSCLE CELL (57) Abstract This invention encompasses a method for inhibiting vascular cellular activity of cells associated with vascular lesion formation in mammals which involves administering an effective dosage of at least one antisense sequence to at least one gene expressing a cyclin or a cyclin dependent kinase which inhibits the expression of the gene. More particularly, the invention involves administering antisense sequences which inhibit the expression of cyclin A, B1, B2, C, D1, D2, D3, E or cyclin X (p46) cyclin X and cyclin dependent kinase cdc2, cdk2, cdk4 or cdk5. It is preferable to use two antisense sequences each from a different cyclin or cyclin dependent kinase. The cyclin or cyclin kinase depending kinase dosage is preferably administered in combination with proliferating cell nuclear antigen (PCNA). Antisense methods and compositions directed to inhibiting the expression of growth factors such as TGF- β ₁ , TGF, bFGF, PDGF are also contemplated by the present invention. The antisense sequences are incorporated into liposomes, particularly liposomes containing HIV and which are directly administered intraluminally, intramurally or periadventitiously. The methods of this invention are useful in treating a broad spectrum of vascular lesions such as lesions in the carotid femoral and renal arteries, particularly lesions resulting from renal dialysis fistulas. The invention is particularly useful in treating vascular grafts <i>ex-vivo</i> and prior to surgical grafting.		

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INHIBITION OF PROLIFERATION OF VASCULAR SMOOTH MUSCLE CELL

BACKGROUND OF THE INVENTION

Technical Field

The field of this invention is inhibition of cellular activity related to vascular lesions.

Background of The Related Art

5 In the growth and maintenance of multi-cellular organisms, the organism has had to develop processes to activate or inhibit the proliferation of cells. The organism has developed numerous mechanisms, whereby signals are given to cells, by either intracellular or extracellular messengers. Control of proliferation provides
10 for modeling of organs, maintenance of subsets of leukocytes in hematopoiesis, wound healing and the like. However, in many situations, such as injury or disease states, the response of the organism to the injury or disease may, in fact, be deleterious to the health of the organism.

The vascular response to injury involves an alteration in three fundamental
15 cellular processes; cell growth, cell migration and extracellular matrix production. This vascular response to injury is characteristic of the pathogenesis of various vascular diseases including (but not limited to): atherosclerosis, restenosis after angioplasty, vein bypass graft stenosis, prosthetic graft stenosis, angiogenesis and hypertension. For example, atherosclerotic lesions evolve as a result of vascular
20 smooth muscle migration into the subintimal space, proliferation and the production of abundant extracellular matrix. Similarly, restenosis after angioplasty, vein bypass graft stenosis, prosthetic graft stenosis, angiogenesis and hypertension involve abnormalities in vascular cell growth, migration and matrix composition. The precise mechanisms responsible for alterations in the regulation of these cellular processes
25 are poorly characterized.

Dzau, Hypertension 8, 553-559 (1986) describes the vascular renin-angiotensin pathway. Geisterfer, et al., Circulation Research 62, 749-756 (1988) report that angiotensin II induces hypertrophy of cultured rat aortic smooth muscle

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cells. Barrett and Benditt, Proc. Natl. Acad. Sci. USA 85, 2810-2814 (1988) describe the expression of platelet-derived growth factor in human atherosclerotic plaques and normal artery wall. Powell, et al., Science 245, 186-188 (1989) report that inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. Naftilan, et al., J. Clin. Invest. 83, 1413-1424 (1989) report the induction of platelet-derived growth factor A chain and c-myc gene expression by angiotensin II in cultured rat vascular smooth muscle cells. Sarzani, et al., J. Clin. Invest. 83, 1404-1408 (1989) describe the expression of various growth factors in aorta of normotensive and hypertensive rats. Majesky, et al., J. Clin. Invest., 88, 904-910 (1991) report the production of TGF- β_1 during repair of arterial injury. Linder, et al., Circulation Research, 68, 106-113 (1991) describe the role of basic fibroblast growth factor in vascular lesion formation. Daemen, et al., Circulation Research, 68, 450-456 (1991) report the role of angiotensin II in inducing smooth muscle cell proliferation in the normal and injured rat arterial wall. Ferns, et al., Science, 253, 1129-1132 (1991) report the inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF, Gibbons, et al., Clin. Research, 38, 287A (1990) report the modulation by transforming growth factor- β of the bifunctional growth response of vascular smooth muscle cells to angiotensin II. Itoh, et al., Biochem. Biophys. Res. Comm., 176, 1601-1609 (1991) report the interaction of atrial natriuretic polypeptide and angiotensin II on protooncogene expression and vascular cell growth.

Simons and Rosenberg, Circ. Res., 70, 835-843 (1992) report that antisense oligonucleotides to non-muscle myosin heavy chain and c-myc suppress smooth muscle cell proliferation *in vitro*. Speir and Epstein, Circulation, 86, 538-547 (1992) report that antisense to proliferating cell nuclear antigen (PCNA) inhibits smooth muscle cell proliferation *in vitro*. Rosenberg et al., PCT/US92/05305, January 1993 describes an antisense approach of localized oligonucleotide therapy involving the inhibition of the c-myc or PCNA protein.

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The relationship of the expression of cyclins at various phases of the cell synthesis and mitosis cycle are described in The Journal of NIH Research, December 1992, Vol. 4, pp. 55-59. Figure 1 for example shows the approximate level of Cyclins A, B1, B2, C, D1, D2 and E during G1 (gap 1), synthesis. G2 (gap 2) and mitotic phase of cell proliferation. Williams, et al., The Journal of Biological Chemistry, Vol. 266, No. 12, April 25 (1993), pp. 8871-8880 shows the isolation of cyclin x (p46).

Both of these later two articles and references such as O'Connor, et al., The Journal of Biological Chemistry, Vol. 286, No. 11, April 15, pp. 8298-8308 (1993) elucidate the interaction of various Cyclin dependent kinases such as cdc2 and cdk2 and Cyclins A and B.

Jaskulski et al., Science, Vol. 240, No. 4858, pp. 1544-6 (1988) describes the effects of antisense oligonucleotides on growing Balb/C313 cells. It was shown that the antisense molecule inhibited synthesis and mitosis and sense oligos had no effect. Sala et al., Proc. Natl. Acad. Sci., USA, Vol. 89, No. 21, pp. 10415-9 (Nov. 1992) describes antisense sequences to c-myc gene which inhibit c-myc expression. Kimeki, J. Cell Biochem, Vol. 50, No. 1, pp. 1-9 (1992) describes antisense interruption of C-myc or Cyclin A *in vitro*. Doyle et al., Antisense Res. Dev., Vol. 1, No. 1 Spring, pp. 11-20 (1991) describes antisense oligonucleotides to Cyclin B in xenopus oocytes. Zindly et al., Biochem Biophys. Res. Commun., Vol. 1897, No. 3, pp. 1144-54 (1992) describes antisense interruption of the production of Cyclin A or Cyclin B in rats. Cyclin A antisense oligonucleotides are also described in Nature, Vol. 354, No. 6351, pp. 314-7 (1991) and Guerra et al., EMBO J., Vol. 10, No. 11, pp. 3343-9 (1991). These references report the effect of Cyclin A or Cyclin B oligos in metaphase I oocytes. Further, antisense studies in xenopus embryos are described in Development, Vol. III, No. 4 pp. 1173-8 (1991) and J. Cell. Biol., Vol. 114, No. 4, pp. 767-72 (1991). Lapidot-Lifson et al., Proc. Natl.

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Acad. Sci., USA, Vol. 89, No. 2, pp. 579-83 (1992) describe the down regulation of cdc2 with antisense oligonucleotides.

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SUMMARY OF THE INVENTION

This invention encompasses a method for inhibiting vascular cellular activity of cells associated with vascular lesion formation in mammals which involves administering an effective dosage of at least one antisense sequence to at least one mRNA or pre-LRNA for a gene for a cyclin or a cyclin dependent kinase which antisense sequence inhibits the expression of the gene. More particularly, the invention involves administering antisense sequences which inhibit the expression of cyclin A, B1, B2, C, D1, D2, D3, E or cyclin X (p46) and cyclin dependent kinase cdc2, cdk2, cdk4 or cdk5. It is preferable to use two antisense sequences each which inhibits expression of a different cyclin or cyclin dependent kinase.

In another aspect, the antisense sequence against cyclin or cyclin dependent kinase is preferably administered in combination with an antisense sequence against proliferating cell nuclear antigen (PCNA).

In yet another aspect of this invention, vein grafts are genetically modified with antisense sequences against cyclin or cyclin dependent kinase prior to surgical grafting.

Antisense methods and compositions directed to inhibiting the expression of growth factors such as transforming growth factor beta (TGF β), basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF) are also contemplated by the present invention.

The antisense sequences are incorporated into liposomes, particularly liposomes containing HVJ and which are directly administered intraluminally, intramurally or periadventitiously.

While the methods of this invention are useful in treating a broad spectrum of vascular lesions. Such lesions include, but are not limited to, lesions in the carotid femoral and renal arteries, particularly lesions resulting from renal dialysis fistulas. The methods of the present invention are particularly useful in treating vascular

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lesions associated with cardiovascular angioplasty. For such use, the antisense sequence is applied to the angioplasty site to reduce restenosis.

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DETAILED DESCRIPTION OF THE INVENTION**Brief Description of the Drawings**

- Figure 1 is a schematic diagram of the appropriate level of various cyclins in G1, S, G2, or M phase states of the cell cycle;
- 5 Figure 2 shows the histology of restenosis;
- Figure 3 shows the effect of combined antisense treatment against cdc2 and PCNA on neointima with time;
- Figure 4 shows the effect of antisense against cdk2 and cdc2 on neointimal/media areas ratio;
- 10 Figure 5 long term efficiency of antisense and sense ODN of cdc2 kinase/PCN on intimal/medial area ratio. $***P < 0.01$ vs. sense.
- Figures 6a, 6b, 6c quantify the PCNA and Cdc2 protein levels and BrdU labeling index respectively in vein grafts;
- 15 Figures 7a, 7b quantify the vein wall thickness and the luminal radius: wall thickness ratio respectively of vein grafts a specific period following surgery; and
- Figure 8 shows the effect of pretreating vein grafts on vein graft tension.

20 The approximate levels of expression of cyclins during the cell cycle are shown in Figure 1, Cyclin D, appears in the gap 1 (G1) phase and is expressed throughout the cycle in the presence of growth factors. Cyclin C accumulates in early G1 and then declines. Cyclin D2 and E accumulate at the end of G1 and are degraded during synthesis (S) phase Cyclin A is first detected in the (S) phase and is degraded as the cells enter mitosis (M). Cyclins B1 and B2 appear soon after cyclin A and are degraded in mid-M phase. These cyclins are important proteins in

25 the cell cycle and are recognized as a family of related proteins by their amino acid

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sequence homology. Cyclins form active complexes with kinases and these active complexes phosphorylate proteins.

Figure 2 illustrates the restenosis process after angioplasty. Angioplasty is performed by inflating a balloon in an artery to relieve an obstructive atherosclerotic lesion. In the course of compressing the lesion the procedure induces tearing of the internal elastic laminae (IEL). Increased blood flow results from relief of the narrowing. The tears in the IEL and injury of the blood vessel wall by the balloon expansion induce a vascular smooth muscle cell proliferative response that results in formation of a neointima and renewed obstruction to the vessel lumen. The neointima consists of smooth muscle cells and matrix and takes 1-6 months to form post angioplasty.

Tables 1 and 2 illustrate sense and antisense sequences for various growth factors, for representative cyclins or, cyclin dependent kinases, and PCNA. Table 3 illustrates various human DNA sequences useful for deriving antisense sequence information for making therapeutic agents of this invention. Table 4 illustrates the sequence of ODN against cell regulatory genes used in various examples. Table 5 are antisense ODN on neointima/medial area ratios as determined in Example 4.

Figures 6a, 6b, and 6c quantify the levels of PCNA and cdc2 protein levels and BrdU labeling index in vein grafts. Specifically, Figure 6a and 6b quantify levels of PCNA and cdc2 kinase protein levels respectively in homogenates of untreated, control ODN treated and antisense ODN treated vein grafts and of contralateral jugular veins measured via ELISA four days after surgery wherein $\dagger p=0.25$, $* p=0.02$. Figure 6c quantifies the level of BrdU labeling indices in vein grafts and contralateral jugular veins one week after surgery wherein $\S p<0.001$

Figures 7a and 7b quantify the wall thickness and luminal radius/wall thickness ratio respectively of the vein grafts at 2, 6 and 10 weeks after surgery in ungrafted veins, control ODN treated vein grafts and antisense ODN treated vein grafts.

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Figure 8 is a plot of the passive length-tension relationships of control ODN treated (o, n=3) and antisense ODN treated (Δ , n=3) vein grafts and of contralateral carotid arteries (\bullet , n=6) and jugular veins (, n=6), 6 weeks after surgery. Tensions were recorded at various lengths and are shown as a function of the percentage increase in length compared to the maximal length achieved for a tension of zero.

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TABLE 1

		<u>Genbank Accession #</u>
	A. human TGF- β_1 MRNA: Nature 316,701-706(1985),	
		X02812
5		J05114
	5'-GCC UCC CCC AUG CCG CCC UCC GGG-3'	
	antisense TGF:	
	3'-GGG TAC GGC GGG AGG-5'	
	control TGF:	
10	sense TGF:	
	5'-CCC ATG CCG CCC TCC-3	
	reverse TGF:	
	5'-GGG TAC GGC GGG AGG-3'	
	B. human BFGF MRNA: N.Y. Acad.Sci. <u>638</u> , 109-123 (1991)	
15	Biochem, Biophys. Res. Commun. <u>144</u> 543-550(1988)	
	(GCA) 5'-GCA GGG ACC AUG GCA GCC GGG AGC-3'	
		M17599
		S81809
	antisense FGF:	
20	3'-CCC TGG TAC CGT CGG-5'	
	control FGF:	
	sense FGF:	
	5'-GGG ACC ATG GCA GCC-3'	
	reverse FGF:	
25	5'-CCC TGG TAC CGT CGG-3'	
	C. human PDGF A chain mRNA:Nature 320, 695-699(1986)	
	5'-CGG GAC GCG AUG AGG ACC UUG GCU-3'	X03795
	antisense PDGF:	

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3'-TAC TCC TGG AAC CGA-5'

control PDGF:

sense PDGF:

5'-ATG AGG ACC TTG GCT-3'

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TABLE 2

	A.	Mouse cdc2 Kinase mRNA	<u>Genbank Accession #</u>
		5'-UGA GUA ACU AUG GAA GAC UAU AUC-3'	X16461
		Antisense	
5		3'-ACT CAT TGA TAC CTT CTG-5'	
		Sense	
		5'-TGA GTA ACT ATG GAA GAC-3'	
	B.	Rat PCNA mRNA	Y00047 and M24604
		5'-AAC UCC GCC ACC AUG UUU GAG GCA GGC CUG-3'	
10		Antisense	
		1: -6 +9	
		3'-CGG TGG TAC AAA CTC-5'	
		2: +4- +21	
		3'-AAA CTC CGT GCG GAC TAG-5'	
15		Sense	
		1: -6 +9	
		5'-GCC ACC ATG TTT GAG-3'	
		2: +4- +21	
		5'-TTT GAG GCA CGC CTG ATC-3'	
20	C.	Rat cyclin B mRNA	X60768
		5'-GGA GCC AUG GCG CUC AGG GUC-3'	
		Antisense Cyclin B	
		3'-CCT CGG TAC CGC GAG TCC-5'	
		Sense Cyclin B	
25		5'-GGA GCC ATG GCG CTC AGG-3'	

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TABLE 3

		<u>Genbank</u>	<u>Accession</u>	<u>#</u>
	A.	Human cdk2 Kinase mRNA, EMBO J 10 2653-2659 (1990)		
				X61622
5		5'-UGG CGC UUC AUG GAG AAC UUC CAA-3'		
		Antisense cdk2:		
		3'-GCG AAG TAC CTC TTG AAG-5'		
		Control		
		Sense cdk2		
10		5'-CGC TTC ATG GAG AAC TTC-3'		
	B.	Human cdc2, <u>Nature</u> 327:31-35 (1987)		X05360
		5'-TTGACTAACTATGGAAGATTATACCAAAATAGAGAAAATT		
		GGAGAAGGTACCTATGGAGTTGTGTATAAGGGT-3'		
	C.	Human cyclin A <u>Nature</u> 343:555-557 (1990)		X51688
15		5'-GGGAGCAGTGATGTTGGGGCAACTCTGCGCCGGGGCCTGCG		
	D.	Human cyclin B <u>Cell</u> 58:833-846 (1989)		M25753
		5'-AGAGGAAGCCATGGCGCTCCGAGTCACCAGGAACTCGAAA		
		ATTAATGCTGAAAATAAGGCGAAGATCAACATG-3'		
	E.	Human cyclin C <u>Cell</u> 66:1197-1206 (1991)		M74091
20		5'-GGCTGGGTCTATGGTCGCTCCGCGGCCGTCCGCCGCGTGG		
		TGCTTTTTTATCAGGGCAAGCTGTGTTCCATGGCAGGGAAC-3'		
	F.	Human cyclin D1 <u>Cell</u> 66:1197-1206 (1991)		M74092
		5'-AGCCCCAGCCATGGAACACCAGCTCCTGTGCTGCGAAGTGG		
		AAACCATCCGCCGCGCGCTACCCCGATGCCAACCTCCTCAAC-3'		
25	G.	Human cyclin D3 <u>J. Biol. Chem.</u> 267:20412-20415		
		(1992)		M92287
		5'-CTGCCCCGAGTATGGAGCTGCTGTGTTGCGAAGGCACCCGGC		
		ACGCGCCCCGGGCCGGGCCGACCCGCGGCTGCTGGG-3'		

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H. Human cyclin E Cell 66:1197-1206 (1991) M74093

5'-GCGGGACACCATGAAGGAGGACGGCGGCGCGGAGTTCTCGG
CTCGCTCCAGGAAGAGGAAGGCAAACGTGACCGTTTTTTG-3'

I. Human PCNA J. Biol. Chem. 264:7466-72 (1989) J04718

5 5'-CTCCGCCACCATGTTCGAGGCGCGCCTGGTCCAGGGCTCCA
TCCTCAAGAAGGTGTTGGAGGCACTCAAGGACCTC-3'

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TABLE 4

Table 4. Sequences of Antisense ODN.

- 5 1) cdc 2 kinase (position -9 to +9 of the mouse sequence)

 Sense 5'-TGA-GTA-ACT-ATG-GAA-GAC-3'

 Antisense 5'-GTC-TTC-CAT-AGT-TAC-TCA-3'
- 2) cdc 2 kinase (position -9 to +9 of the rat sequence)

 Sense 5'-TGA-GTA-ACT-ATG-GAG-GAC-3'

 Antisense 5'-GTC-TTC-CAT-AGT-TAC-TCA-3'
- 10 3) PCNA (position +4 to +22 of the rat sequence)

 Sense 5'-TTT-GAG-GCA-CGC-CTG-ATC-3'

 Antisense 5'-GAT-CAG-GCG-TGC-CTC-AAA-3'
- 4) cdk 2 kinase (position -6 to +12 of human sequence)

 Sense 5'-CGC-TTC-ATG-GCG-AAC-TTC-3'

 Antisense 5'-GAA-GTT-CTC-CAT-GAA-GCG-3'
- 15 5) cyclin B1 (position of -9 to +9 of rat sequence)

 Sense 5'-GAA-GGA-GCC-ATG-GCG-CTC-3'

 Antisense 5'-GAG-CGC-CAT-GGC-TCC-TCC-3'
- 6) Scrambled ODN

 5'-CTT-CGT-CGG-TAC-CGT-CTT-C-3'
- 20 7) thrombomodulin

 Antisense 5'-ACC-CAG-AAA-GAA-AAT-CCC-3'

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Table 5. Effect of antisense ODN on neointima/medial areas ratio

	SENSE	ANTISENSE	UNTRANSFECTED
PCNA	n.t.	1.460 ± 0.193	n.t.
cdc3 kinase			
mouse	1.250 ± 0.188	0.741 ± 0.126*	1.244 ± 0.253
rat	n.t.	0.814 ± 0.201**	1.853 ± 0.208
cdk 2 kinase	1.341 ± 0.307	0.597 ± 0.131**	1.156 ± 0.077
cdc 2 (mouse) + PCNA	1.174 ± 0.235	0.084 ± 0.06**	1.510 ± 0.203
cdc 2 (rat) + PCNA	n.t.	0.130 ± 0.074**	0.931 ± 0.27
cdc 2 (rat) + cyclin B1	n.t.	0.267 ± 0.029**	1.220 ± 0.189
cdc 2 (mouse) + cdk 2	1.440 ± 0.366	0.283 ± 0.188**	n.t.
scrambled	1.695 ± 0.136		
thrombomodulin	n.t.	1.551 ± 0.099	1.625 ± 0.183
injured (vehicle)	1.759 ± 0.398		
injured (untreated)	1.432 ± 0.188		

* P < 0.05. ** P < 0.01 vs. sense treated and/or untransfected vessels.

n.t. = not tested, vehicle = treated with HVJ complex without ODN, N = 4-8 rats.

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The invention is related to a method for inhibiting expression of protein-encoding genes using antisense oligonucleotides. The method is based on the localized application of the oligonucleotides to a specific site *ex-vivo*. The oligonucleotides preferably are applied directly to the target tissue in a mixture with
5 an implant or gel, or by direct injection or infusion. In one aspect, the oligonucleotides are modified to render them resistant *ex-vivo* to degradation or alteration by endogenous enzymes.

The therapeutic approach using antisense oligonucleotides is based on the principle that the expression of a protein from a gene can be down regulated by
10 providing an appropriate length oligonucleotide which is complementary to at least a portion of the messenger RNA (mRNA) transcribed from the gene. The antisense strand hybridizes with the mRNA and targets the mRNA for down regulation thereby preventing ribosomal translation, and subsequent protein synthesis.

Preferred Oligomers

15 The Oligomer selected may be any of a number of types, including those having a charged or uncharged backbone.

Preferred Oligomers include alkyl- and aryl- phosphate Oligomers, especially preferred are methylphosphonate Oligomers. Other preferred Oligomers include phosphorothioate Oligomers, phosphorodithioate Oligomers, morpholino
20 analogs, formacetal analogs, thioformacetal analogs and peptide nucleic acid ("PNA") analogs.

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Preferably the Oligomers each comprise from about 4 to about 40 nucleosides, more preferably, from about 6 to 30 nucleosides. Especially preferred are Oligomers of about 8 to about 20 nucleosides.

5 Preferred target regions when the nucleic acid target sequence is mRNA or pre-mRNA include the 3'-untranslated region, the coding region, splice sites of pre-mRNA (including splice donor and splice acceptor sites), the initiation codon region including regions slightly downstream of the AUG start codon (preferably up to about 20 nucleotides downstream from the AUG initiation codon), regions immediately 5'- of the initiation codon (preferably about 5 to 200 nucleotides 5'- of the AUG start
10 codon) and the 5'-cap cite.

According to an alternately preferred aspect, tandem Oligomers are employed. Preferred tandem Oligomers include those which comprise a total of about 20 to about 40 nucleosides.

15 Oligomers having the selected internucleoside linkages may be conveniently prepared according to synthetic techniques known to those skilled in the art. For example, commercial machines, reagents and protocols are available for the synthesis of Oligomers having phosphodiester and certain other phosphorus-containing internucleoside linkages. See also Gait, M.J., Oligonucleotide Synthesis: A Practical Approach (IRL Press, 1984); Cohen, Jack S., Oligodeoxynucleotides Antisense Inhibitors of Gene Expression, (CRC Press, Boca Raton, FL, 1989); Oligonucleotides and Analogues: A Practical Approach, (F. Eckstein, 1991); and Agarwal, S.,
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Protocols for Oligonucleotides and Analogs Synthesis and Properties, (Humana Press, N.J., 1993). Preparation of Oligomers having certain non-phosphorus-containing internucleoside linkages is described in United States Patent No. 5,142,047, the disclosure of which is incorporated herein by reference.

5 Synthetic methods for preparing methylphosphonate Oligomers are described in Lee B.L., et al., Biochemistry 27:3197-3203 (1988), Miller, P.S., et al., Biochemistry 25:5092-5097 (1986), and "An Improved Method for the Synthesis and Deprotection of Methylphosphonate Oligonucleotides" in Protocols for Oligonucleotides and Analogs Synthesis and Properties, S. Agarwal, ed. (Humana Press, N.J. 1993); and published PCT applications WO 92/07864 and WO 92/07882
10 the disclosures of which are incorporated herein by reference.

 Also preferred are Oligomers which are nucleoside/non-nucleoside polymers. Suitable Oligomers also include chimeric oligonucleotides which are composite RNA, DNA analogues (Inouo et al., FEBS Lett. 2115:327 (1987)). Other suitable
15 Oligomers include Oligomers having chimeric backbones. Such chimeric backbone Oligomers include Oligomers having mixed phosphate backbones including nucleoside sequences which are capable of activating RNaseH, nucleoside sequences which do not activate RNaseH, and thus allow site directed cleavage of an RNA molecule. See U.S. Patent No. 4,149,797 which is incorporated herein by reference. Chimeric
20 backbone Oligomers also include Oligomers having a mixture of internucleosidyl linkages which may or may not include phosphorus atoms, such as morpholinyl

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linkages, formacetal linkages, peptide nucleic acid (PNA) linkages and the like. Oligomers having a neutral backbone, for example, methylphosphonate Oligomers with cleaving or cross-linking moieties attached, may prove advantageous in certain circumstances; such Oligomers may have a longer half-life *ex-vivo* since the neutral structure reduces the rate of nuclease digestion while the cleaving or cross-linking moiety may promote inactivation of target polynucleotide sequences.

According to one aspect of the present invention, these antisense Oligomers have a sequence which is complementary to a portion of the RNA transcribed from the selected target gene. Although the exact molecular mechanism of inhibition has not been conclusively determined, it has been suggested to result from formation of duplexes between antisense Oligomer and the RNA transcribed from the target gene. The duplexes as formed may inhibit translation, processing or transport of an mRNA sequence or may lead to digestion by the enzyme RNaseH.

Single stranded Oligomers may also bind to a duplex DNA target such that a duplex is formed with one of the two DNA strands, and the second DNA of the target strand is displaced from the duplex. Preferred is the formation of a duplex by the Oligomer with the coding strand of the DNA duplex target ("invading duplex"). The invading duplex so formed may inhibit transcription.

As a general matter, the Oligomer employed will have a sequence that is complementary to the sequence of the target nucleic acid. However, absolute complementarity may not be required; in general, any Oligomer having sufficient

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complementarily to form a stable duplex with the target nucleic acid is considered to be suitable. Since stable duplex formation depends on the sequence and length of the hybridizing Oligomer and the degree of complementarity between the antisense Oligomer and the target sequence, the system can tolerate less fidelity (complementarily) when longer Oligomers are used. However, Oligomers of about 8 to about 40 nucleosidyl units in length which have sufficient complementarity to form a duplex having a melting temperature of greater than about 40°C under physiological conditions are particularly suitable for use according to the method of the present invention.

Definitions

As used herein, the following terms have the following meanings unless expressly stated to the contrary.

The term "purine" or "purine base" includes not only the naturally occurring adenine and guanine bases, but also modifications of those bases such as bases substituted at the 8-position, or guanine analogs modified at the 6-position or the analog of adenine, 2-amino purine, as well as analogs of purines having carbon replacing nitrogen at the 9-position such as the 9-deaza purine derivatives and other purine analogs.

The term "nucleoside" includes a nucleosidyl unit and is used interchangeably therewith, and refers to a subunit of a nucleic acid which comprises a 5-carbon sugar and a nitrogen-containing base. The term includes not only those nucleosidyl units

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having A, G, C, T and U as their bases, but also analogs and modified forms of the naturally-occurring bases. In RNA, the 5-carbon sugar is ribose; in DNA, it is 2'-deoxyribose. The term nucleoside also includes other analogs of such subunits, including those which have modified sugars such as 2'-O-alkyl ribose.

The term "phosphate" refers to the group $O=P-R$



Wherein R is hydrogen or an alkyl or aryl group. Suitable alkyl or aryl groups include those which do not sterically hinder the phosphate linkage or interact with each other. The phosphate group may exist in either an "R" or an "S" configuration. Phosphate groups may be used as internucleosidyl phosphorus group linkages (or links) to connect nucleosidyl units.

The term "phosphodiester" or "diester" refers to the group $O=P-O$



wherein phosphodiester groups may be used as internucleosidyl phosphorus group linkages (or links) to connect nucleosidyl units.

A "non-nucleoside monomeric unit" refers to a monomeric unit wherein the base, the sugar and/or the phosphorus backbone has been replaced by other chemical moieties.

A "nucleoside/non-nucleoside polymer" refers to a polymer comprised of nucleoside and non-nucleoside monomeric units.

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The term "oligonucleotide" or "Oligomer" refers to a chain of nucleosides which are linked by internucleoside linkages which is generally from about 4 to about 100 nucleosides in length, but which may be greater than about 100 nucleosides in length. They are usually synthesized from nucleoside monomers, but may also be obtained by enzymatic means. Thus, the term "Oligomer" refers to a chain of oligonucleosides which have internucleosidyl linkages linking the nucleoside monomers and, thus, includes oligonucleotides, nonionic oligonucleotide alkyl- and aryl-phosphate analogs, alkyl- and aryl- phosphonothioates, phosphorothioate or phosphorodithioate analogs of oligonucleotides, phosphoramidate analogs of oligonucleotides, neutral phosphate ester oligonucleotide analogs, such as phosphotriesters and other oligonucleotide analogs and modified oligonucleosides, and also includes nucleoside/non-nucleoside polymers. The term also includes nucleoside/non-nucleoside polymers wherein one or more of the phosphorus group linkages between monomeric units has been replaced by a non-phosphorous linkage such as a formacetal linkage, a thioformacetal linkage, a sulfamate linkage, or a carbamate linkage. It also includes nucleoside/non-nucleoside polymers wherein both the sugar and the phosphorous moiety have been replaced or modified such as morpholino base analogs, or polyamide base analogs. It also includes nucleoside/non-nucleoside polymers wherein the base, the sugar, and the phosphate backbone or a nucleoside are either replaced by a non-nucleoside moiety or wherein a non-nucleoside moiety is inserted into the nucleoside/non-nucleoside polymer. Optionally, said non-nucleoside moiety may serve to link other small molecules which may interact with target sequences or alter uptake into target cells.

The term "alkyl- or aryl-phosphate Oligomer" refers to Oligomers having at least one alkyl- or aryl-phosphate internucleosidyl linkage. Suitable alkyl- or aryl- phosphate groups include alkyl- or aryl- groups which do not sterically hinder the phosphate linkage or interact with each other. Preferred alkyl groups include lower alkyl groups having from about 2 to about 6 carbon atoms. Suitable aryl

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groups have at least one ring having a conjugated pi electron system and include carbocyclic aryl and heterocyclic aryl groups, which may be optionally substituted and preferably having up to about 10 carbon atoms.

5 The term "methylphosphonate Oligomer" (or "MP-Oligomer") refers to Oligomers having at least one methylphosphonate internucleosidyl linkage.

10 The term "neutral Oligomer" refers to Oligomers which have nonionic internucleosidyl linkages between nucleoside monomers (i.e., linkages having no positive or negative ionic charge) and include, for example, Oligomers having internucleosidyl linkages such as alkyl- or aryl- phosphate linkages, alkyl- or aryl-
15 phosphonothioates, neutral phosphate ester linkages such as phosphotriester linkages, especially neutral ethyltriester linkages; and non-phosphorus-containing internucleosidyl linkages, such as sulfamate, morpholino, formacetal, thioformacetal, and carbamate linkages. Optionally, a neutral Oligomer may comprise a conjugate between an oligonucleotide or nucleoside/non-nucleoside polymer and a second
20 molecule which comprises a conjugation partner. Such conjugation partners may comprise intercalators, alkylating agents, binding substances for cell surface receptors, lipophilic agents, nucleic acid modifying groups including photo-cross-linking agents such as psoralen and groups capable of cleaving a targeted portion of a nucleic acid, and the like. Such conjugation partners may further enhance the uptake of the Oligomer, modify the interaction of the Oligomer with the target
25 sequence, or alter the pharmacokinetics distribution of the Oligomer. The essential requirement is that the Oligonucleotide or nucleoside/non-nucleoside polymer that the Oligomer conjugate comprises be substantially neutral and capable of hybridizing to its complementary target sequence.

The term "substantially neutral" in referring to an Oligomer refers to those Oligomers in which at least about 80 percent of the internucleosidyl linkages between the nucleoside monomers are nonionic linkages.

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The term "neutral alkyl- or aryl- phosphate Oligomer" refers to neutral Oligomers having neutral internucleosidyl linkages which comprise at least one alkyl- or aryl- phosphate linkage.

5 The term "neutral methylphosphonate Oligomer" refers to neutral Oligomers having internucleosidyl linkages which comprise at least one methylphosphonate linkage.

10 The term "tandem oligonucleotide" or "tandem Oligomer" refers to an oligonucleotide or Oligomer which is complementary to a sequence located either on the 5'- or 3'- side of a target nucleic acid sequence and which is co-hybridized with a second Oligomer which is complementary to the target sequence. Tandem Oligomers may improve hybridization of these Oligomers to the target by helping to make the target sequence more accessible to such Oligomers, such as by decreasing the secondary structure of the target nucleic acid sequence. In addition, one member of a pair of tandem Oligomers may improve the hybrid stability of the second tandem
15 Oligomer to the target nucleic acid sequence by promoting a helical structure at either the 5'- or 3'- end of said second Oligomer and vice-versa.

A preferred therapy is a combination of antisense molecules targeted against a cyclin and a cyclin dependent kinase. By "antisense molecules against", "antisense sequences against", "antisense against", and similar terms is meant an oligomer
20 complementary to a nucleic acid target sequence for a particular cyclin or cyclin dependent kinase which oligomer will prevent or interfere with expression or function of the particular cyclin or cyclin dependent kinase. Certain combinations of cyclins and cyclin dependent kineses that are reported to be involved in smooth muscle proliferation and restenosis. In the transition of cells from G1 to S-phase, the early
25 stages of smooth muscle proliferation in response to injury in the vessel wall, the kinase cdk2 is reported to be activated by a combination of cyclins A and E which appear late in G1 (Koff, et al., Science, 1992 257:1689). Accordingly, a combination preferred of antisense molecules to decrease or inhibit restenosis would

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be either a combination of antisense oligomers against cyclin A and cdk2 or cyclin E and cdk2. Cyclin P46 or cyclin X (Williams et al. J. Biol. Chem., 1993 268:8871) has been reported to appear in mid G1 and to activate cdk2 ahead of cyclins A and E. Thus, another preferred combination would be the combination of antisense against cyclin X and cdk2. The cell cycle dependent kinase cdk4 has been reported to be activated earlier in G1 than in cdk2, and appears to be important in the G1-S-phase transition. Cdk4 has been reported to be activated by the inductions of cyclins D2 and D3. (Matsushime, et al. Cell, 1992. 71:323). We believe the combinations of antisense sequences against D2 and cdk4, or D3 and cdk4 are also preferred.

Once cells pass through the G1-S-phase transition and replicate their DNA, they are termed "competent". Cell division can be inhibited by preventing synthesis or conversely by inhibiting the later stage transition from G2 to M-phase termed "mitosis". Cdc2 kinase controls "progression" and it is activated by complexing with the cyclins B1, B2, and A. (Parker and Pinwoica-Worms, Science, 1992. 257:1955). Thus, a preferred combinations of antisense oligomers to inhibit mitosis would be a cyclin B - cdc2, or a cyclin A-cdc2. Since the induction of cyclins has been reported as a key event in activation of cyclin-kinase complex leading to transition of cells into and through the cell cycle (Ohtsubo and Roberts, Science, 1993, 259:1908). Cyclins D-type, D2 and D3 which have been reported to activate cdk4 (Ajchenbaum et al., J. Biol. Chem., 1993, 268:4113) are reported to be induced by growth factors. Accordingly, antisense oligomers to these proteins may be employed according to the methods of the present invention.

The net effect of induction of cyclins is the activation of the nuclear kinase complexes leading to phosphorylation of nuclear proteins and then liberation of free transcription factors such as E2F that promote expression of genes that are important in replication of DNA and mitosis. O'Connor et al. (J. Biol. Chem., 1993. 268:8298) have reported that the nitrogen mustard drugs, known inhibitors of cell division, work by specifically interfering with the cyclin A-cdk2 kinase complex and

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the cyclin B1-cdc2 kinase complex. It is believed that combinations of antisense molecules which interfere with the cyclin A-cdk2 complex will prevent DNA synthesis and molecules that interfere with the cyclin B1-cdc2 complex will interfere with mitosis.

5 A preferred combination is an antisense sequence against proliferating cell nuclear antigen (PCNA) and an antisense sequence to cyclin or a cyclin dependent kinase and most particularly an antisense sequence against PCNA and cdc2. Antisense sequences complimentary to the mRNA of the proteins that express basic fibroblast growth factor (bFGF), transforming growth factor-beta (TGF- β), and
10 platelet derived growth factor (PDGF) alone or in combination with the above cyclins or cyclin dependent kinases are useful in practicing this invention.

Methods of Application of the Oligonucleotides

The antisense sequences may be administered in any convenient vehicle, which is physiologically acceptable. For the most part liquid media will be employed, such
15 as saline, phosphate buffered saline, aqueous ethanol, or other media composition compatible with the oligomers and the tissue or cells to be treated. In some instances liposomes may be employed to facilitate uptake of the antisense oligonucleotide into the target vascular smooth muscle cells, particularly where the liposome is bound to ligands for target cell receptors, which provide for endocytosis of the subject
20 compositions. Hydrogels may also be used as depots for placement at the site of the lesion. See, for example, WO 93/01286.

Of particular interest is the use of cationic liposomes for use in cationic liposome-mediated transfection (lipofection) (see Itoh, et al. Hypertension 16, 325 (abstract) (1990)) or neutral liposomes. Concentrations of liposome will generally
25 range from about 1-5, preferably 2-4 $\mu\text{g/ml}$ in the medium, where the ratio of liposome to DNA will generally be about 1:3-1:10 (w/w), preferably about 1:6 (w/w). Neutral liposomes also find application, where mono or diester glycerides (fatty acids of from 8-24 carbon atoms, saturated or unsaturated), particularly

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phosphatidyl serine, phosphatidyl choline, etc., cholesterol, or other neutral liposome-forming monomers may find use. Moreover, complexing or covalent binding to the liposomes of proteins which aid in the binding and uptake of the liposomes find use, such as viral coat proteins, monoclonal antibodies to surface membrane proteins, and the like. The manner of binding and the particular proteins will vary depending on the target tissue, manner of administration, protein availability, stability, level of immunogenicity and the like.

The invention is useful in preventing proliferation of smooth muscle cells in a variety of vascular procedures such as coronary artery angioplasty, renal artery angioplasty, carotid artery surgery, renal dialysis fistulae stenosis, and vascular graft stenosis.

The antisense sequence is in a pharmaceutically acceptable compositions and is applied directly to the vascular site of injury.

Kato, et al., The Journal of Biological Chemistry, Vol. 266, No. 6, Feb. 25, pp. 3361-3364 (1991), Kaneda et al., Science, Vol. 243 pp. 375-378 (1988) describe liposomes containing the hemagglutinating virus of Japan (HVJ) as a vehicle for delivering DNA to cells. The HVJ containing liposomes have been found to be particularly useful vehicles for localized delivery of the antisense sequences of this invention.

Whenever possible, administration of the subject compositions will be localized, either by virtue of cell surface recognition markers, or by the manner of administration, such as catheter, syringe, placement of a depot, and the like. In some instances, the subject compositions may be infused upstream from the site of the cells whose activity is to be modulated. The agent can be introduced to the vessel wall lumenally, intramurally or periadventitally to localize the agent at the site of the lesion. One preferred method is to deliver the antisense drug through a catheter placed in the coronary artery at the site of the lesion following the angioplasty. The localized concentration or amount administered may be determined empirically and

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will depend upon the purpose of the administration, the area to be treated, the effectiveness of the composition, the manner of administration, and the like. The local concentration will desirably be in the range of about 0.1 to 50 μ M.

5 Antisense oligonucleotides of this invention can also be applied to vein grafts using an intraoperative gene therapy approach to block medial smooth muscle cell proliferation thereby preventing accelerated atherosclerosis that is often responsible for vein graft failures. Such *ex-vivo* gene therapy redirects vein graft biology away from neointimal hyperplasia and toward medial hypertrophy thereby producing grafts that closely resemble normal arteries in structure and function. Furthermore, the
10 genetically engineered grafts are resistant to diet-induced atherosclerosis.

Vein graft remodeling without gene therapy results in a reduction of the high distensibility of veins and decreases wall stress to normal arterial levels. This process forms a neointimal layer of smooth muscle cells (SMC) that is highly susceptible to accelerated atherosclerosis. In fact, up to 50% of vein bypass grafts
15 fail within a period of 10 years as a result of this occlusive disease. The genetic engineering therapy of this invention induces vein grafts to follow a different path of arterial adaptation by which mechanical stability is achieved via medial hypertrophy, thereby mimicking arterial structure and decreasing the grafts' susceptibility to atherosclerosis.

20 Vein grafts respond to two major stimuli: 1) injury due to manipulation and ischemia at the time of surgery, and 2) the increased intraluminal pressure and shear stress exerted on the venous wall by the arterial environment. Injury to the vessel wall promotes neointima formation in arteries as well as veins, and exposure to increased pressure in the absence of vascular injury can induce vascular hypertrophy without neointima formation. Since vascular SMC in G_0/G_1 phase can undergo
25 cellular hypertrophy and produce extracellular matrix proteins without progressing through the cell cycle, the blockade of gene expression necessary for the transition of SMC to the DNA replication phase by using antisense oligonucleotides of this

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invention permits vascular hypertrophy of vein grafts and inhibits the SMC proliferation and migration involved in neointimal hyperplasia. Antisense oligonucleotide (ODN) blockade of expression of cell division cycle 2 (*cdc2*) kinase and proliferating cell nuclear antigen (PCNA) prevents neointimal hyperplasia in engineered "arterialized" reversed interposition jugular vein grafts in mammals using, for example, a hemagglutinating virus of Japan (HVJ)-liposome *ex-vivo* transfection technique. The genetically modified grafts demonstrate a sustained resistance to diet-induced atherosclerosis.

The following examples are offered by way of illustration and not by way of limitation of the present invention.

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EXPERIMENTAL

Example 1

Growth of Vascular Smooth Muscle Cells in Culture

5 Rat aortic smooth muscle cells (passage 5-10) isolated and cultured according to the method of Owens, et al. (J. Cell. Biol., 102, 343-352 [1986]), were plated into 24-well culture dishes at 1×10^4 cells/well. At confluence, the cells were made quiescent by incubation for 48 hours in a defined serum-free (DSF) medium containing insulin (5×10^{-7} M), transferrin ($5 \mu\text{g/ml}$), and ascorbate (0.2 mM). This growth condition maintains smooth muscle cells in a quiescent, non-catabolic state and promotes the expression of smooth muscle cell-specific contractile proteins.

Synthesis and Purification of Oligomers

15 Oligonucleotide sequences utilized in this study and their relationships to TGF- β_1 , bFGF, and PDGE A chain mRNAs (Sporn, et al., J. Cell Biol. 105, 1039-1045 [1987]; Burgess and Maciag, Ann. Rev. Biochem. 58, 575-606 [1989]; Betsholz, et al., Nature 320, 695-699 [1986] are shown below.

20 Unmodified, 15-base deoxyribonucleotides were synthesized on an automated solid-phase synthesizer (Applied Biosystems Incorporated) using standard phosphoramidite chemistry. Prior to use, the oligomers were purified by gel filtration, ethanol-precipitated, lyophilized to dryness and dissolved in the culture media. Antisense TGF, antisense FGF and antisense PDGF oligonucleotides were complementary to human TGF- β_1 mRNA, bFGF and mRNA, and PDGR A chain mRNA, respectively, at the translation initiation region. Control oligonucleotides were either the sense oligonucleotide (sense TGF, sense FGF, sense PDGF) or the oligonucleotide with the same oligonucleotide sequence but with a reversed 5'-3' orientation (reverse TGF, reverse FGF). To introduce the oligonucleotides into VSMC, a cationic liposome-mediated transfection method (lipofection) was employed (Itoh, et al. Hypertension 16, 325 [abstract] [1990]; Nabel, et al., Science 249, 1285-1288 [1990]). Oligonucleotides dissolved in 50 μl DSF media were mixed with

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Lipofectin™ Reagent DOTMA (N[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride) (BRL Life Technologies, Gaithersburg, MD) dissolved in the same volume of water in a ratio of 6/1 (w/w) and incubated for 30 minutes at room temperature. The oligonucleotides/liposome complex (100 µl) was then added dropwise to each well.

Bioassay for TGF-β

CCL-64 mink lung epithelial cells (Danielpour, et al., J. Cell. Physiol. 138, 79-86 [1989]) were maintained in MEM supplemented with 10% fetal calf serum and 0.1 mM nonessential amino acids. Cells were spread at a density of 4×10^4 cells/well in 24-well plates one day before the assay. The subconfluent cells were washed once and fed with DSF containing vehicle or TGF-β (human TGF-β₁, R & D Systems, Minneapolis, MN). Twenty hours later, the cells were pulsed for 8 hours with ³H-thymidine (10 µ Ci/ml). The incorporation of ³H-thymidine was determined as described below and expressed as the percent of incorporation of the control (without TGF-β) wells. The levels of TGF-β, secreted from quiescent VSMC in a 14-hour period were similarly assayed at four dilutions.

Demonstration that the inhibitory effects of the conditioned media were due to TGF-β was accomplished by blocking the growth inhibitory effect with a neutralizing antibody. Fresh DSF media, human TGF-β₁ (2 ng/ml) in DSF media, or the conditioned media collected from VSMC were incubated at 37°C for 1 hour with either turkey preimmune serum or turkey anti-human TGF-β₁ antiserum (Danielpour, et al., 1989, *supra*) (1/200 final dilution prior to the addition to the CCL-64 mink lung epithelial cell bioassay at a 1/2 dilution. This treatment completely abolished the growth inhibitory action of the conditioned media (Gibbons, et al., Clin. Res. 38, 287a [1990]).

Bioassay for bFGF

Extraction of bFGF from VSMC and bioassay for bFGF activity using mouse 3T3 fibroblasts were performed as in a previous report (Klagsbrun, et al., Proc. Natl.

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Acad. Sci. USA 83. 2448-2452 [1986]). Confluent quiescent rat VSMC (1.3×10^7) with or without previous treatment with antisense FGF-oligonucleotides were harvested from monolayer cultures by trypsinization, washed with phosphate-buffered saline, (1 μ g/ml), pepstatin (4 μ M) and phenylmethylsulfonyl fluoride (1 nM). After cells were disrupted by 3 cycles of freezing and thawing followed by sonication for 1 minute, the homogenate was centrifuged at 25,000xg for 30 minutes and the supernatant was dialyzed overnight against 0.1 M NaCl/0.01 M Tris-HCl, pH 7.5. All procedures were performed at 4°C, and aliquots of cell extracts were stored at -80°C until use. For the measurement of bFGF activity, human bFGF standards 0.03-3 ng/ml, Genzyme Corporation, Boston, MA) or samples pre-incubated (2 hours at 37°C) with either anti-bFGF IgG (R & D Systems, Minneapolis, MN) or non-immune IgG at 10 μ g/ml were incubated with quiescent Swiss 3T3 cells for 20 hours, after which the cells were pulse-labeled with 10 μ Ci/ml 3 H-thymidine for 8 hours. Addition of non-immune IgG had no effect on basal or bFGF-stimulated thymidine incorporation, nor did the administration of anti-bFGF IgG affect basal thymidine uptake. Anti-bFGF IgG (10 μ g/ml) almost completely abolished the mitogenic activity of 1 ng/ml recombinant human bFGF, without affecting the mitogenic activity of acidic FGF or PDGF. Serial dilution curves of cell extracts were parallel to the standard curve of bFGF, and cellular bFGF content was estimated by antibody-suppressible mitogenic activity in the samples.

Determination of DNA Synthesis

Relative rates of DNA synthesis were assessed by determination of tritiated thymidine (10 μ Ci/ml) incorporated into trichloroacetic acid (TCA)-precipitable material as previously reported (Itoh, et al., J. Clin. Invest. 86, 1690-1697 [1990]).

Results

Ang II was shown to induce a 2.5-fold increase in the VSMC synthesis and release of biologically-active TGF- β using the mink lung epithelial cell bioassay. In this assay, active TGF- β caused a dose-dependent inhibition of DNA synthesis of the

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cells ($IC_{50} = 8 \times 10^{-11}$ M). The growth inhibitory activity in the conditioned media of the VSMC could be abolished by prior incubation of the media with a specific TGF- β neutralizing antibody. This demonstrated the specificity of the assay for TGF- β .

5 The synthesis by VSMC of bFGF was shown to be stimulated by Ang II. Messenger RNA for bFGF was induced modestly by Ang II (approximately 2-fold), while antibody-inhibitable bFGF activity in cell extracts, as detected by the Swiss 3T3 cell bioassay was increased 3-fold. In this assay, confluent, quiescent VSMC are exposed to vehicle (basal) or Ang II (10^{-6} M) and the cells extracted and assayed for antibody-inhibitable bFGF activity using Swiss 3T3 fibroblasts as bioassay. The standard curve demonstrated the dose-dependent stimulation of DNA synthesis by bFGF. Extracts from Ang II-treated cells contained 2-3-fold higher bFGF activity.

10 Antisense oligonucleotides (15 mer) complementary to human TGF- β_1 , bFGF and PDGF A chain mRNAs as well as control oligonucleotides were synthesized. See Fig. 1.

15 The oligonucleotides were introduced into VSMC by cationic liposome-mediated transfection (lipofection). The optimum *ex-vivo* concentration of cationic liposome and its ratio to DNA that minimized cell toxicity and optimized DNA uptake were determined to be 2-4 μ g/ml and 1:6 (w/w), respectively.

20 To determine the effect of antisense oligonucleotides on the production of TGF- β and bFGF, confluent quiescent VSMC were treated with antisense or control oligonucleotides (5 μ M) directed against TGF- β or bFGF. 50 and 250 μ l of conditioned media were collected after 20 h and assayed for TGF- β activity using the mink lung epithelial cell bioassay. Antisense oligonucleotides resulted in a 75% decrease in TGF- β activity. bFGF content in extracts of control or antisense oligomer-treated VSMC was assayed using the Swiss 3T3 bioassay. The antisense oligomer inhibited bFGF production by 85%.

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Extracts from quiescent VSMC contained 2.9 ng of bFGF per mg protein. This quantity was unaffected by incubation with control oligonucleotide but was decreased to below detectable levels (< 1 ng/mg protein) when the cells were incubated with 4 μ M antisense oligomer for 24 h.

5 In a companion study, the growth of cultured endothelial cells, which utilized bFGF as an autocrine growth factor, was also inhibited effectively with antisense oligomers directed against bFGF.

10 In the next study, the effects of the antisense TGF- β oligonucleotide on DNA synthesis in basal and Ang II-stimulated VSMC were determined. The effect of antisense oligomers on VSMC proliferation was determined by transfecting confluent quiescent VSMC with antisense or control oligonucleotides (5 μ M) 4 hours prior to addition of vehicle or Ang II (10^{-6} M). After 20 hours, the cells were labeled with 10 μ Ci/ml 3 H-thymidine for 8 hours. Thymidine incorporation was then determined by measuring the radioactivity of the cells. TGF- β antisense oligomer (5 μ M)
15 potentiated DNA synthesis by 35% in basal state, but more significantly in Ang II-stimulated state (87%, $p > 0.05$). In contrast, there was no change in the rate of DNA synthesis in Ang-II-stimulated cell groups transfected with the control oligomer. The results indicate that TGF- β exerts a tonic inhibitory action on VSMC proliferation and that in the Ang II-stimulated state, it plays an even greater role in
20 growth inhibition. The antisense TGF treatment also causes a significant reduction of RNA synthesis (3 H-uridine incorporation) by 60% at the basal state. These results support the conclusion that endogenous production of TGF- β exerts a hypertrophic and antiproliferative action.

25 The effect of the antisense oligomer to bFGF employed at 5 μ M was shown to suppress DNA synthesis in the basal state and inhibited this process significantly by (30%) in the Ang II-stimulated state. bFGF acts as a promoter of VSMC proliferation, especially in the Ang II-stimulated state. In contrast, antisense oligomers directed against PDGF-A had no effect on basal or Ang II-stimulated 3 H-

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thymidine incorporation. The data supports the conclusion that Ang II activates a growth-stimulatory pathway mediated primarily by bFGF.

Co-transfection of bFGF antisense oligomer with TGF- β antisense oligomer almost completely abolished Ang II-induced VSMC proliferation. The co-transfection of bFGF antisense oligomer with TGF- β antisense oligomer almost completely abolished Ang II VSMC proliferation that was unmasked by the blockade of TGF- β production. In contrast, transfection of the antisense directed against PDGF-A had no effect on the DNA synthesis uncovered by the antisense oligomer directed against TGF- β .

The above data supports the conclusion that the bFGF and TGF- β growth factor counteract within the same population in an autocrine and/or intracrine fashion. Since bFGF is known to promote the production of plasminogen activator, which is crucial for the activation of TGF- β , the interaction of these dual autocrine loops in the production and activation of these growth factors is an essential aspect of the regulation of VSMC proliferation.

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Example 2

Confluent, quiescent cells, cultured as described in Example 1, were exposed to a combination of antisense oligomers directed against cdc2 kinase, cdk2 kinase, and cyclin B (Tables 2 and 3) (4 μ M each). The oligomers were synthesized as described above with the exception that phosphorothiate modified nucleotides were employed. The oligomers (antisense and control) were introduced into the cells using virally-mediated liposome uptake (Kaneda, et al., J. Biol. Chem., (1989) 264, 12126-12129). To assess the effect of these oligomers on the growth of VSMC, cells were grown for 4 days after oligomer exposure. The cells were then harvested by trypsin and assayed for DNA content. The results demonstrated that sense oligomer treated cultures had DNA contents indistinguishable from control untreated cultures, demonstrating that the sense oligomers had no effect on the basal growth of the VSMC cultures. On the other hand, cells exposed to the antisense oligomers exhibited a 50% decrease in DNA content, demonstrating that these oligomers decreased by 50% the basal growth of these cultures.

These oligomers also inhibited growth factor stimulated growth. Treatment of these cells with FGF increased the content of DNA by 4-fold. In cultures treated with FGF plus sense oligomers, DNA content increased 3-fold, demonstrating that the presence of sense oligomers had little effect on growth factor-induced increase in DNA content. However, when FGF-treated cells were exposed to the antisense oligomers, no increase in the basal levels of DNA were observed, demonstrating that these oligomers blocked the growth factor induced growth of these cells.

Similar results were seen when a combination of antisense oligomers directed against cdc2 kinase and PCNA were used.

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Example 3Growth of Vascular Smooth Muscle *Ex-vivo* in Response to Injury

Male Sprague-Dawley rats weighing 500-550 grams were used. Endothelial denudation and injury to the vascular wall were performed on carotid arteries. Rats were anesthetized with the administration of ketamine hydrochloride (80 mg/kg) (Parke-Davis, Morris Plains, NJ) and xylazine (12 mg/kg) (Lloyd Laboratories, Shenandoah, IA). A 2 French embolectomy balloon catheter (American Edward Laboratories, Santa Ana, CA) was passed into the left common carotid via the left external carotid artery according to the previously described method of Clowes, et al. (Lab Invest. 49, 208-215 [1983]). Using Evans Blue staining, we documented that this procedure denuded the endothelium effectively. The right, uninjured carotid was used as a control.

At the time of injury, some animals received a combination of antisense oligomers directed against cdc2 kinase and PCNA (tables 2 and 3). The synthesis of the oligomers was as described above except that phosphothionate modified sequences were employed. The oligomers were introduced lumenally to the vessel wall. Uptake of the DNA was mediated via virally coated liposomes (Kaneda, et al., J. Biol. Chem. 264, 12126-12129 [1989]).

To determine the effect of the oligomer treatment on DNA synthesis in the injured vessel, the animals were injected with BrdUr. This thymidine analogue is incorporated into the newly synthesized DNA and is detected immunohistochemically.

All rats were euthanized 4 days following injury. Rats were anesthetized and blood was withdrawn from the ascending aorta via the left ventricle. The rats were then perfused at a pressure of 110 mm/Hg through the ascending aorta with 100 ml of phosphate buffered saline (PBS, pH 7.2). The carotid arteries were removed and small segments were fixed in 10% formaldehyde and embedded in paraffin. Five micron sections were cut on a rotary microtome and placed on glass slides that had been treated for 20 seconds with acetone containing 2% 3-aminopropyltriethoxysilane

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(Sigma Chemical, St. Louis, MO). The sections were digested with 0.1% trypsin (Sigma Chemical, St. Louis, MO) in PBS at 37°C for 12 minutes. The sections were then preincubated with normal serum from the species that the second step antibody was produced. The sections were then covered with a primary antibody specific for BrdUr, incubated for 15 minutes at 4°C, washed with PBS and then incubated with the biotinylated secondary antibody. After washing, the sections were incubated with avidinbiotinylated horseradish peroxidase complex (Vectastain ABC kits, Vector Laboratory, Burlingame, CA), and developed with 0.1% diaminobenzidine tetrahydrochloride (Aldrich Chemical, Milwaukee, WI) and 0.02% H₂ O₂ (Sigma Chemical, St. Louis, MO), the slides were then counter stained with hematoxylin. Positive staining appeared as brown in color. Negative controls included the following: 1) omission of primary or secondary antibody, 2) replacement of the primary antibody by non-immune serum or immunoglobulin of the same species.

The results demonstrated that control uninjured vessels exhibit very low levels of DNA synthesis (<1% BrdUr labeling index). On the other hand, the injured untreated vessel inhibited a high level of BrdUr staining (23% BrdUr labeling index) demonstrating that vascular injury induces DNA synthesis within the vessel wall. In the cases of exposure of these injured vessels to antisense oligomers, DNA synthesis was decreased by 60%. Moreover, DNA content within the antisense treated vessel wall decreased by 40% when compared to the sense treated vessel.

Neointima formation was assessed by morphometry of perfusion-fixed specimens 14 days after balloon injury in a blinded fashion. Our results demonstrated a dose-dependent suppression of neointima formation by the combination of PCNA-cdc2 antisense oligodeoxynucleotide (ODN). A low dose (3 µM each) of antisense ODN inhibited neointima formation by approximately 60% (n=7) compared to vessels treated with the HVJ-liposome complex alone (n=5) or sense ODN-treated vessels (n=8). The antisense ODN had no significant effect on medial area. At a dose of 15 µM each, a single administration of the PCNA-cdc2

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antisense ODN combination completely abolished neointima formation (n=8), (greater than 95%) whereas sense control ODN had no effect (n=8) Figure 3. Importantly, a significant inhibition of neointima formation was observed up to 4 and 8 weeks after administration of the PCNA-cdc2 antisense ODN combination compared to sense control, Figure 3. Intraluminal transfection of PCNA antisense ODN alone failed to inhibit neointima formation (neointimal area= 0.237 ± 0.016 mm² versus sense control area= 0.214 ± 0.022 mm², p. 0.05). These data suggest that there is an amplified inhibitory effect of combined administration of both PCNA and cdc2 antisense ODN. The selectivity of the antisense ODN effect was further confirmed by the observation that the inhibition of the neointima was limited to the area of intraluminal transfection. In contrast, the adjacent injured carotid segments outside the area of antisense transfection exhibited neointimal lesions similar to the sense ODN-treated control. These results are summarized in Figure 3.

The intraluminal administration of antisense cdk2 alone at 3 μ M and 15 μ M with the HVJ method resulted in a 60% reduction in DNA synthesis as measured by BrdUr labeling as well as a 60% reduction in neointima lesion formation at 2 weeks after vascular injury (Figure 4, columns 4 and 6). As can be seen from Figure 4, column 5, the effect of administering the combination of cdk2 antisense ODN (3 μ M) and cdc2 (3 μ M) antisense ODN on neointima formation was not significantly different from cdk2 (3 μ M) alone.

Since DNA synthesis in the cell of the injured vessel is required for full development of the neointimal lesion, inhibition of DNA synthesis in those cells should inhibit the lesion formation.

The above concentrations of antisense sequences to PCNA and cdc2 were conveniently used to demonstrate the synergistic effect of these sequences on modifying DNA synthesis and cardiovascular cellular activity of vascular smooth muscle cells associated with formation of neointima. The desired range of antisense concentration can be determined empirically for difference antisense sequences and

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will vary depending on factors such as, but not limited to, the nature and location of the sequence targeted by the antisense and the length of the antisense sequence.

Example 4

This example summarizes a study performed in an attempt to enhance the efficiency of cellular uptake and the stability of antisense ODN. The study involves a viral protein-mediated ODN transfer technique.

Synthesis of oligomers and selection of sequence targets

The sequences of antisense oligonucleotides (ODN) against cell cycle regulatory genes used in this Example are shown in Table 4. Synthetic ODN were washed by 70% ethanol, dried, and dissolved in sterile Tris-EDTA buffer (10mM Tris, 1 mM EDTA.) The supernatant was purified over NAP 10 column (Pharmacia), and quantitated by spectrophotometry as described in Itoh, J., Mukoyama, M., Pratt, R.e., Dzau, V.J. (1992) Biochem Biophysic Res Comm 188, 1205-1213.

Preparation of HVJ-liposomes

Phosphatidylserine, Phosphatidylcholone, and cholesterol were mixed in a weight ratio of 1:4.8:2 as described in Kaneda, Y., Iwai, K., Uchida, T. (1989) Science 243, 375-378; Tomita, N., Higaki, J., Morishita, R., Kato, K., Mikami, J., Kaneda Y., Ogihara, T. (1992) Biochem Biophysic Res Comm 186, 129-134; Morishita, R., Gibbons, G.H., Kaneda, Y., Ogihara, T., Dzau, V.J., J Cell Biochem; Itoh, J., Mukoyama, M., Pratt, R.e., Dzau, V.J. (1992) Biochem Biophysic Res Comm 188, 1205-1213. Dried lipid was hydrated in 200 μ l of balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6) containing sense or antisense-ODN (600 nmol). Liposomes were prepared by shaking and sonication. Purified HVJ (Z strain) was inactivated by UV irradiation (110 erg/mm²/sec) for 3 min just before use. The liposome suspension was mixed with HVJ (10000 hemagglutinating units) in a total volume of 4 ml of BSS. The mixture

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was incubated at 4°C for 5 minutes and then for 30 minutes with gentle shaking at 37°C. Free HVJ was removed from the HVJ-liposomes by sucrose density gradient centrifugation. The top layer of the sucrose gradient was collected for use. The final concentration of antisense ODN is equivalent to 15 μ M, as calculated according to Kaneda, Y., Iwai, K., Uchida, T. (1989) Science 243, 375-378; Tomita, N., Higaki, J., Morishita, R., Kato, K., Mikami, J., Kaneda Y., Ogihara, T. (1992) Biochem Biophys Res Comm 186, 129-134; Morishita, R., Gibbons, G.H., Kaneda, Y., Ogihara, T., Dza, V.J., J Cell Biochem.

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Ex-vivo transfer of ODN

A 2 French Fogarty catheter was used to induce vascular injury in male Sprague-Dawley rats (400-500g; Charles River Breeding Laboratories) as described in Rakugi, J., Jacob, J.K., Krieger, J.E., Ingelfinger, J.R., Pratt, R.E., (1993) Circulation 87, 283-290. The rats were anesthetized, and a cannula introduced into the left common carotid via the external carotid artery. After vascular injury of the common carotid, the distal injured segment was transiently isolated by temporary ligatures. The HVJ-liposome complex was infused into the segment and incubated for 10 minutes at room temperature. After a 10 minute incubation, the infusion cannula was removed. Following the transfection, blood flow to the common carotid was restored by release of the ligatures. No adverse neurological or vascular effects were observed in any animal undergoing this procedure.

At 2, 4 and 8 weeks after transfection, rats were sacrificed and vessels were perfusion-fixed with 4% paraformaldehyde. Three individual sections from the middle of transfected segments were analyzed. In addition, three sections from the middle section of the injured untransfected region was also analyzed. Animals were coded so that the operation and analysis were performed without knowledge of which treatment individual animals received.

A statistical analysis of the results is found in Table 5. All values are expressed as mean \pm SEM. All experiments were repeated at least three times. Analysis of variance with subsequent Duncan's test was used to determine significant differences in multiple comparisons. $P < 0.05$ was considered significant.

Initially, the effect of a single antisense ODN at 15 μ M on the inhibition of neointima formation in rat carotid artery model was examined. Administration of either antisense cdc 2 kinase (both mouse and rat sequences) or antisense cdk 2 kinase ODN resulted in the partial inhibition of neointima formation after balloon injury, whereas antisense PCNA ODN alone failed to show any inhibitory effects. To achieve the further inhibition a combination of these ODN were administered.

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Indeed, a single administration of 15 μ M of the PCNA/cdc 2 (mouse sequence) antisense ODN combination completely abolished neointima formation (n=8), whereas the sense control ODN (15 μ M each) had no effect (n=8). The antisense ODN had no significant effect on medial area. The administration of combination of cdc 2 kinase (rat sequences) and PCNA ODN inhibited neointima formation almost completely.

Also examined was the combination of antisense cdc 2 kinase and cyclin B ODN. A single administration of combination of antisense cdc 2 and cyclin B ODN inhibited neointima formation significantly. The selectivity of the antisense ODN effect was further confirmed by the observation that the inhibition of the neointima formation was limited to the area of intraluminal transfection along the injured carotid artery. (Table 4). In contrast, the adjacent injured carotid segments outside the area of antisense transfection exhibited neointimal lesions similar to the sense ODN-treated control injured carotid artery. Transfection of antisense thrombomodulin ODN also failed to show any inhibitory effects on neointima formation. These results summarize Table 5.

The single administration of 15 μ M of the PCNA/cdc 2 antisense ODN combination significantly inhibited the extent of neointima formation for a period of 8 weeks after transfection (Fig. 5). Although there was evidence for a neointimal lesion at 4 weeks after antisense treatment, the lesion was significantly inhibited as compared to sense treatment ($P < 0.01$). Furthermore, no further progression of the lesion was observed at 8 weeks after a single antisense administration.

The results of this example indicates that neointima lesion formation after balloon angioplasty can be prevented by a single intraluminal administration of antisense oligonucleotides directed against cell cycle regulatory genes. This therapeutic strategy fulfills the criteria needed for success, *i.e.*, a proper drug target, an efficient drug delivery method and an intraluminal approach.

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In defining the strategy to prevent restenosis in humans, the method of drug delivery is critically important. Intraluminal delivery is desirable since it can be carried out concomitantly with the transluminal angioplasty --a practical issue of clinical relevance. The blockade of cell cycle progression is particularly attractive since it maintains the cells in a quiescent, differentiated phenotype without inducing cell injury. The HVJ-OFN intraluminal method substantially increases the efficiency of uptake and the stability of ODN *ex-vivo* thereby avoiding the potential toxicity of high concentrations of ODN (*i.e.* > 150 μ M). The modification of antisense ODN pharmacokinetics by use of the HVJ-liposome complex will facilitate the potential clinical utility of these agents by: 1) allowing for an abbreviated intraluminal incubation time to preserve organ perfusion, 2) prolonging the duration of biological action, 3) enhancing specific activity.

Example 5

This example examines the fate of antisense oligodeoxynucleotides (ODN) *ex-vivo* using high efficient viral mediated transfer method (HVJ). Direct transfer of unmodified FITC-labeled ODN showed fluorescence in the medial layer, and disappeared within 1 day. In contrast, transfected unmodified FITC-ODN by HVJ method showed much fluorescence in the medial layer, and fluorescence continued at least 1 week (and disappeared within 2 weeks). Moreover, transfer of phosphorothioate FITC-ODN enwrapped in liposomes without HVJ particle resulted in a short existence of fluorescence (within 4 days), while transfer by HVJ method result in continuous fluorescence up to 2 weeks after transfection. Given that the HVJ method prolonged the half-lives of ODN the efficiency of antisense phosphorothioate ODN against cdk kinase gene to balloon injury rat carotid artery was also examined. The specificity of antisense cdk 2 ODN was confirmed by the observation that mRNA of cdk 2 kinase in injury vessels was markedly diminished by the antisense ODN treatment. At 2 weeks after transfection, antisense cdk 2 ODN treatment resulted in

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a significant inhibition in neointima formation, while sense ODN treatment and untreated did not result in reduction. On the other hand, administration of antisense cdc 2 ODN also partially inhibited neointima formation. Moreover, a single intraluminal administration of combination of antisense cdc 2 and cdk 2 ODN showed further neointima inhibition as compared to antisense cdc 2 ODN alone. This example demonstrates that a single intraluminal administration of antisense ODN directed cell cycle regulatory genes such as cdc 2 and cdk 2 kinase with HVJ method prevented neointima formation after balloon angioplasty in rat carotid injury model via a catheter delivered system.

Synthesis of oligomers and selection of sequence targets

The sequences of ODN against human cdk 2 kinase used in this study was following: antisense: 5'-GAA-GTT-CTC-CAT-GAA-GCG-3', sense: 5'-CGC-TTC-ATG-GAG-AAC-TTC-3' (-6 - +12 of human sequence; these sequences are no different between mouse and human cdk 2 kinase). This antisense ODN specifically inhibit cdk2 kinase protein synthesis and serum stimulated growth in rat VSMC. Also synthesized were sense and antisense cdc 2 kinase ODN (antisense: 5'-GTC-TTC-CAT-AGT-TAC-TCA, sense: 5'-TGA-GTA-ACT-ATG-GGA-GAC-3', -9 to +9 of mouse sequence), as described in Furukawa, U., Piwnica-Worms, H., Ernst, T.J. Kanakura, Y., Griffin, J.J.D. (1990) Science 250, 805-808. Synthetic ODN were purified over NAP 10 column (Pharmacia), and quantitated by spectrophotometry.

Preparation of HVJ-liposomes

Phosphatidylserine, Phosphatidylcholine, and cholesterol were mixed in a weight ratio of 1:4.8:2. Dried lipid was hydrated in 200µl of balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, 10mM Tris-HCl, pH7.6) containing sense of antisense-ODN (120 nmol). Control group was used as no ODN (BSS 200 ul). Liposomes were prepared by shaking and sonication. Purified HVJ (Z strain) was

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inactivated by UV irradiation (100 erg/mm²/sec) for 3 min just before use. The liposome suspension (0.5 ml, containing 10 mg of lipids) was mixed with HVJ (10000 hemagglutinating units) in a total volume of 4 ml of BSS. The mixture was incubated at 4°C for 5 min and then for 30 min with gently shaking at 37°C. Free HVJ was removed from the HVJ-liposomes by sucrose density gradient centrifugation. The final concentration is equivalent to 3 μ M. In this study, the preparation of HVJ complex was optimized to achieve the maximum effect of antisense ODN in VSMC.

Ex-vivo gene transfer

A 2 French Fogarty catheter was used to induce vascular injury in male Sprague-Dawley rats (400-500g; Charles River Breeding Laboratories). These rats were anesthetized with ketamine, and the left common carotid artery was surgically exposed. A cannula was introduced into the common carotid via the external carotid artery. *Ex-vivo* gene transfer was assessed under following condition: Vascular injury of the common carotid was induced by the passage and inflation of a balloon catheter through an arteriotomy in the external carotid artery three times. The injured segment was transiently isolated by temporary ligatures. The HVJ-liposome complex, liposome complex without HVJ particles, or ODN alone was infused into the segment and incubated for 10 minutes at room temperature as described above. After a 10 minute incubation, the infusion cannula was removed. Following the transfection, blood flow to the common carotid was restored by release of the ligatures, and the wound was then closed. No adverse neurological or vascular effects were observed in any animal undergoing this procedure.

Ex-vivo transfection of FITC labeled ODN

FITC labeled unmodified and phosphorothioate antisense ODN were kindly provided by Clontech Inc. (Palo Alto, CA.) FITC was labeled to 3' and 5' of ODN

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(16 mer). Animal surgery was performed as described above. Transfer of unmodified ODN was performed under two protocols. (1) HVJ complex with FITC labeled unmodified ODN ($3\mu\text{M}$) was incubated for 10 minutes. (2) ODN alone: unmodified ODN $30\mu\text{M}$ was incubated for 10 minutes. Transfer of phosphorothioate ODN was also performed under two protocols; (1) HVJ complex with FITC labeled phosphorothioate ODN ($3\mu\text{M}$) was incubated for 10 minutes. (2) ODN in liposomes without HVJ particles: liposome complex with ODN ($3\mu\text{M}$), but without HVJ particles was incubated for 10 minutes. The vessels were harvested at 10 min, 1, 4, 7 and 14 days after transfection and fixed with 3% paraformaldehyde after perfusion of PBS. Sections were examined by fluorescent microscopy, after stained for 5 min in erichrome black T solution and washed twice for 3 min in PBS. Elastic fibers stained dark red and are readily distinguishable from the specific FITC ODN by the treatment by erichrome black T solution.

Reverse transcription (RT)-PCR

RNA was extracted from antisense or sense ODNs (cdk 2 kinase; $15\mu\text{M}$ wrapped in liposomes) treated injured vessels by RNazol (Tel-Test, Inc., Texas) at 1 and 14 days after transfection. Contralateral arteries were also used in intact arteries. Levels of cdk 2 and beta-actin mRNAs were measured by reverse transcription-PCR. The cdk 2 5' primer (neucleotides -6-12 of human sequences) was 5'-CGCTTCATGGAGAACTTC-3'; the 3' primer (neucleotides 340-357) was 5'-ATGGCAGAAAGCTAGGCC-3'. The primers complementary to the rat beta-actin gene were 5'-TTGTAACCAACTGGGACGATATGG-3'. The primers complementary to the rat beta-actin gene were 5'-TTGTAACCAACTGGGACGATATGG-3'; the 3' primer was 5'-GATCTTGATCTTCATGGTGCTAGG-3' (Clontech Laboratories Inc., Palo Alto, CA). Extreme care was taken to avoid contamination of tissue samples with trace amounts of experimental RNA. Aliquots of RNA derived from intact and injured

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vessels were amplified simultaneously by PCR (35 cycles) and compared with a negative control (primers without RNA). Amplification products were electrophoresed through 2% agarose gels stained with ethidium bromide.

Ex-vivo transfection of antisense ODN

5 After balloon injury as described above, 500 μ l of HVJ-liposomes complex containing sense or antisense cdk 2 kinase or cdc 2 kinase ODN (3 or 15 μ M wrapped in liposome) was incubated within lumen for 10 minutes. Direct transfer (cdk 2 ODN; 150 μ M) and liposome-mediated transfer without HVJ particle (cdk 2 ODN; 15 μ M) were also examined to rat injured carotid arteries. At 2 weeks after
10 transfection, rats were sacrificed and vessels were perfusion-fixed with 4% paraformaldehyde. Neointima formation was assessed by morphometry, by individuals who were blinded to the identity of the samples. Intimal and medial areas were measured on a digitizing tablet (southern Micro Instruments, model 2200, Atlanta, GA).

15 *Statistical analysis*

All values are expressed as mean \pm SEM. All experiments were repeated at least three times. Analysis of variance with subsequent Duncan's test was used to determine significant differences in multiple comparisons. $P < 0.05$ was considered significant.

20 **RESULTS**

Localization of FITC labeled unmodified and phosphorothioate ODN

Direct transfer of FITC unmodified ODN (30 μ M) without HVJ method into rat carotid arteries revealed florescence in the medial layer at 10 minutes after transfection. However, this florescence disappeared 1 day after transfection. In
25 contrast, transfer of FITC unmodified ODN (3 μ M) by HVJ method resulted in much

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wider florescence than direct transfer without HVJ method at 10 min after transfection despite of much lower concentration of ODN. Moreover, the florescence was stimulated in the nuclei and continued up to 1 week after transfection. At 2 weeks after transfection, florescence could not be observed in the medial layer.

5 Florescence in ODN-liposome (3 μ M) without HVJ particles transfected vessels was mainly seen in the media layer, but also in the adventitia at 10 min after transfection, similar to direct transfer. This florescence was markedly diminished at 1 day after transfection and disappeared within 4 days. Transfer of FITC labeled phosphorothioate ODN (3 μ M) by HVJ method resulted in much wider florescence
10 which was seen at the medial and adventitial layer, and continued up to 2 weeks. This prolongation of florescence was due to the modification of backbone of ODN to phosphorothioate because of their resistance to nuclease. These findings support the utility of phosphorothioate ODN in antisense strategy. At 1 and 2 weeks after transfection, florescence was mainly found in the medial layer, but not in the
15 neointima lesions. The florescence could be observed in the nuclei at this stage. Untreated of HVJ complex without ODN treated vessels revealed no specific florescence, except autofluorescence in elastic lamina. Florescence resulted by FITC labeled ODN was easily distinguished from autofluorescence. Incubation of free FITC in the vessels did not also result in specific florescence suggesting that this
20 florescence was specific to FITC labeled ODN. Table 6 summarize these results.

Table 6. Summary of FITC labeled ODN transfection

<u>time-course</u>	<u>direct</u>	<u>HVJ-</u> <u>unmodified</u>	<u>liposome</u>	<u>HVJ-S</u>
10 min	+	+	+	+
1 days	nd	+	\pm	+
4 days	nd	+	nd	+

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7 days		+		+
14 days		nd		+

nd = not detected, \pm = few florescence, + = florescence

direct = direct transfer (30 μ M), HVJ-unmodified = unmodified ODN by HVJ method (3 μ M), liposome = liposome mediated transfer of phosphorothioate ODN (3 μ M) without HVJ particle, HVJ-S = phosphorothioate ODN (3 μ M) by HVJ method.

Reverse-transcriptase PCR of cdk 2 kinase mRNA expression ex-vivo

mRNA expression of cdk 2 kinase in rat carotid injury model was examined to evaluate the specificity of antisense cdk 2 ODN. RNA from either the vessels transfected antisense or sense cdk 2 ODN (15 μ M) was amplified by RT-PCR. At 1 day after injury, RT-PCR of RNA from injured vessels transfected with sense ODN demonstrated a single band corresponding to cdk 2 kinase mRNA, whereas from intact vessels did not. However, administration of cdk 2 ODN abolished the increased mRNA. At least 14 days after injury, RT-PCR of RNA from vessels transfected neither sense nor antisense ODN demonstrated the detectable level of cdk 2 mRNA. In contrast, mRNA of beta-actin was readily detected in the same protocol.

Effect on antisense cdk 2 and cdc 2 kinase ODN

Antisense cdk 2 kinase phosphorothioate ODN was applied to rat injured vessels given the phosphorothioate ODN could be stabled within 2 weeks in medial layer by HVJ transfer method. Untreated and sense ODN (2 and 15 μ M) transfected vessels exhibited neointimal formation at 2 weeks after transfection. In contrast, a single administration of antisense cdk 2 kinase ODN (3 μ M) resulted in a significant reduction in neointima formation (approximately 60% as to compared

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to sense ODN treated vessels). At high dose of 15 μ M, antisense cdk 2 ODN resulted in a further inhibition of neointima formation, but not significant. Those treatment did not alter the medial areas, suggesting that media viability was not affected by HVJ transfer method. This reaction in neointima formation was limited in transfected lesions, but not in untransfected lesions in the same animals (neointimal/medial areas ratio; transfected region = 0.597 ± 0.131 VS untransfected region - 1.156 ± 0.079 , $P < 0.01$). To investigate the contribution of HVJ transfer to the enhanced lives of ODN *ex-vivo*, the biological action of antisense cdk 2 ODN among the direct transfer, liposome mediated transfer without HVJ particle, was compared to the HVJ method. Neither direct transfer (150 μ M) nor liposome mediated transfer without HVJ particle (15 μ M) failed to show any inhibitory effect of antisense ODN.

(1) antisense cdc 2 kinase ODN alone (15 μ M), and (2) combination of cdc 2 and cdk 2 kinase ODN (15 μ M each) were administered investigate the antisense strategy blocking cell cycle regulatory genes. Administration of antisense cdc 2 kinase ODN partially inhibited neointima formation as well as cdk 2 ODN alone, whereas sense cdc ODN did not. The combination of cdc 2 and cdk 2 kinase ODN showed enhanced inhibition of neointima formation as compared to antisense cdc 2 or cdk 2 kinase ODN alone.

This example demonstrates that HVJ method prolonged the existence of florescence in the medial layer up to 1 week (unmodified ODN) and 2 weeks (phosphorothioate ODN) after transfection of FITC-labeled ODN, respectively. These findings indicate that the HVJ method bypasses endocytosis. This is probably due to the anticipation of HVJ particles, but not to liposomes, as ODN-liposome complex without HVJ particles did not result in such a prolongation. Moreover, the stimulation of florescence in the nuclei that could be seen only by HVJ method may increase the specificity of antisense ODN.

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Example 6

Preparation of HVJ-liposome oligonucleotide complexes. HVJ-liposome complexes were formed as described in Morishita et al., *Proc. Natl. Acad. Sci. USA*, 90: 8474-78 (1993). Briefly, a mixture of phosphatidyl serine, phosphatidyl choline and cholesterol (1:5:2 by weight) was coated onto a glass tube by evaporation of tetrahydrofuran. Solutions of phosphorothioate ODN (Genta, San Diego, CA) or TITC labeled phosphorothioate ODN (3' and 5' end labeled via fluorescein-ON phosphoramidite, (Clontech, Inc., Palo Alto, CA) (150 uM of each ODN) in balanced salt solution (BSS - 137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH7.6) were introduced, and liposomes were formed via agitation and sonication. After incubation at 37°C for 30 minutes, 10,000 hemagglutinating units of purified, UV-inactivated HVJ (Z strain) were added to the liposomes. The mixture was incubated for 10 minutes at 4°C and then for 30 minutes at 37°C. Separation of unincorporated viral particles was achieved by ultracentrifugation over a sucrose gradient, and 2 uM CaCl₂ and 1 mM glucose were added to the HVJ-liposome complex just prior to transfection. The final concentration of ODN in the liposome complexes was 15 uM.

The following sequences of phosphorothioate ODN were used: antisense cdc2 (position -9 to +9) (5'-GTCTTCCAT-AGTTACTCA-3'); four base pair mismatch antisense cdc2 (5'-GTCTGCCGTCGTTAGTCA-3'); antisense PCNA (position +4 to +22) (5'-GATCAGGCGTGCCTCAAA-3'); four base pair mismatch antisense PCNA (5'-GATTAGTCG-TACCTAAAA-3'). Sense and reverse antisense sequences were also used as controls. Sequences are based on rat PCNA and cdc2 genes; although the rabbit sequences have not been cloned, a 98% homology across species has been documented for these genes. Transfection of rabbit vascular SMC *in vitro* with these antisense and control ODN confirmed sequence specific inhibition of cdc2 kinase and PCNA protein expression and of cellular proliferation in this species. Assuming that the rat sequences may represent a one or two base pair mismatch with

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the rabbit sequences, we further verified the sequence specificity of this antisense ODN effect by confirming that ODN based on the human sequences (antisense cdc2: 5'-ATCTTCCATAGTTAGTCA-3'; antisense PCNA: 5'-GACCAGGCGCGCCTCGAA-3') had equivalent efficacy in preventing rabbit vascular SMC proliferation *in vitro*.

Vein graft model and ex-vivo transfection. New Zealand White rabbits (2.5-3 kg) were anesthetized with an intravenous mixture of ketamine (2.5 mg/ml) and xylazine (0.09 mg/lm). A 2-3 cm segment of right jugular vein was dissected via a midline vertical neck incision, and all side branches were carefully ligated with 4-0 silk suture and divided. The distal vein was ligated just above its bifurcation, and was cannulated via its external branch with a 24 gauge catheter, flushed with normal saline and clamped proximally. Approximately 500 ul of HVJ-liposome complex were infused generating a pressure of approximately 100 mm Hg, and were allowed to incubate for twenty minutes. The animal was then heparinized (200 units/kg), and the vein segment was harvested, rinsed in normal saline and anastomosed into the divided ipsilateral carotid in a reverse end to end fashion using interrupted 7-0 prolene sutures. Papaverin was applied topically (15 mg), and the wound was closed with 3-0 nylon suture.

Some animals were fed a diet of 1% cholesterol (Dyets, Inc., Bethlehem, PA) for one week prior to grafting, and were maintained on that diet until vein graft harvest at two and six weeks. Serum cholesterol was found to reach a level between 800 and 2000 mg/dl, and remained within this range for the period between grafting and harvest. Animal care complied with the "Principles of Laboratory Animal Care" and the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 8-23, revised 1985).

PCNA and cdc2 kinase protein levels. Animals were sacrificed at four days for measurement of PCNA and cdc2 protein levels in vein graft tissue homogenates. The vein grafts and contralateral jugular vein were flushed with normal saline.

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dissected free of surrounding scar and connective tissue, and homogenized via polytron. PCNA and cdc2 protein levels were then measured using an ELISA kit (Paracelsian, Ithaca, NY).

5 BrdU labeling index. BrdU labeling was undertaken at one week after grafting. Animals were treated with BrdU at 18 hours (100 mg/kg subcutaneous and 30 mg/kg intravenous) and 12 hours (30 mg/kg intravenous) prior to harvest. Evans blue dye (25 mg/kg) was infused in some animals one hour prior to sacrifice. Vein grafts were perfusion fixed at 100 mm Hg with 4% paraformaldehyde, dehydrated, and imbedded in paraffin. Contralateral jugular veins were harvested as controls.
10 Five micron sections were stained with a BrdU labeling kit (Amersham, Buckinghamshire, England). Nuclei within the media and neointima staining positive for BrdU were counted, and total medial and neointimal nuclei were counted on adjacent hematoxylin-eosin stained sections to derive a labeling index. Four sections of each vein graft spaced at approximately 0.75 mm intervals were analyzed in this way and an average labeling index for each vessel was obtained.
15

Vessel morphometry. Vein grafts and ungrafted jugular veins were perfusion fixed at 100 mmHg and harvested at two weeks, six weeks and 10 weeks and paraffin-imbedded sections were stained with hematoxylin-van Geison or with Verhoeff-van Geison stain. Computerized planimetry (South Micro Instruments, Model 220, Atlanta, GA) was used to measure intimal and medial cross sectional surface areas on four sections of each vessel spaced at 0.75 mm intervals. Media was differentiated from adventitia by the pink staining of adventitial collagen. Neointima was distinguished by its position relative to the internal elastic lamina and by its chaotic organization of cells compared to the circumferential arrangement of medial SMC. Lumenal radius and intimal and medial thicknesses were derived from these data, and averages for each vessel were calculated.
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Vessel DNA and protein content. Grafts at two weeks and six weeks were carefully dissected free of surrounding scar and connective tissue, homogenized via

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polytron and analyzed for DNA content (Pierce, Rockford, IL) and protein content (Biorad, Hercules, CA).

Immunohistochemistry. Grafts from animals fed a 1% cholesterol diet were perfusion fixed and processed to paraffin, and 5 micrometer sections were taken at 0.75 mm intervals along each graft for immunohistochemical staining with RAM-11, a monoclonal antibody for rabbit macrophages (Dako, Carpinteria, CA) using a biotin-avidin immunoperoxidase procedure. Adjacent sections were also stained with hematoxylin-van Geison.

Length-tension Measurements. At six weeks after surgery, untreated grafts, reverse antisense treated grafts, antisense treated grafts and contralateral carotid arteries and jugular veins were harvested and dissected free of adventitia and scar tissue, and 4 mm rings (3 from each vessel) were suspended in an organ chamber between hooks attached to a tensiometer. Passive tensions were recorded at various lengths and compared to the maximal length achieved for a tension of zero. EDTA was used in a calcium free physiologic solution to eliminate active tension in the vessel walls.

Transfection of fluorescent (FITC)-labeled ODN into rabbit jugular vein graft walls using the HVJ-liposome preparation revealed that transfection of a vein segment *ex-vivo* with an intraluminal incubation period of 20 minutes and a distending pressure of 100 mm Hg just prior to grafting resulted in a consistent, uniform uptake and nuclear localization of ODN in medial cells within 1 hour after implantation that was sustained for over 1 week. Evans blue dye exclusion demonstrated that the endothelium was intact in control and antisense transfected vein grafts at one week post-operatively. Patency was maintained in approximately 90% of all grafts, and this rate was not affected by antisense or control ODN transfection.

PCNA and cdc2 kinase protein levels increased 10- and 5-fold, respectively, in both untreated and control ODN transfected vein grafts four days after grafting, and an increase in DNA replication in control vein grafts was similarly documented

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by bromodeoxyuridine (BrdU) labeling at 1 week (Figures 6a, 6b, 6c). Transfection of the vein grafts with antisense ODN abolished the increase in expression of these cell cycle proteins by over 90%, and markedly inhibited the increase in the number of cells undergoing DNA replication within the graft.

5 Morphometric analysis of the vein grafts revealed that antisense ODN treatment had, in fact, shifted the adaptation of vein graft architecture. Both untreated and control ODN transfected grafts had a total wall thickness approximately 6 times those of ungrafted veins at 6 and 10 weeks, with a neointima/media ratio of about 1.5:1 (Fig. 7a). In contrast, the blockade of PCNA and cdc2 kinase protein
10 expression was effective to yield a marked inhibition of neointima formation at 2 weeks that persisted up to 10 weeks after surgery. During that interval, however, antisense ODN treated grafts underwent medial hypertrophy, such that the medial thickness of these grafts nearly approximated the overall thickness of control grafts that exhibited extensive neointimal hyperplasia. Minimal neointimal formation was
15 seen on some microscopic sections of antisense treated grafts, but the majority of sections examined remained entirely free of this cellular accumulation. The media of antisense treated vessels consisted of SMC organized in their normal circumferential architecture and was delimited by an internal elastic lamina, in contrast to the marked cellular disarray that characterized the neointima of untreated
20 and control ODN treated grafts.

 By the sixth week after grafting, untreated and control transfected vein grafts were found to have lumenal radius/wall thickness ratios, proportional to wall stress, that approximated the values measured in adjacent carotid arteries. The medial hypertrophy seen in antisense transfected vein grafts by 6 weeks similarly succeeded
25 in reducing this ratio to near arterial levels (Figure 7b). The high distensibility of ungrafted veins was also found to be shifted in both control and antisense treated grafts, which displayed length-tension relationships closer to the carotid artery than to the jugular veins from which they were derived (Figure 8). Furthermore,

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biochemical evidence of vascular hypertrophy was reflected in the increase in protein/DNA ratio of antisense ODN treated grafts from 103 ± 2 at 2 weeks to 229 ± 39 at 6 weeks, compared to the ratios of 210 ± 27 observed in carotid arteries and 86 ± 9 and 89 ± 8 in control ODN treated and untreated grafts at 6 weeks, respectively (n-4).

Within two weeks after grafting in hypercholesterolemic rabbits, foam cells identified immunohistochemically as macrophages were observed in untreated and control ODN transfected grafts, primarily at the intima-media interface. By six weeks large numbers of these cells were found throughout the intima of these grafts and in subendothelial accumulations that corresponded to areas of macroscopic plaque. However, no plaque formation was observed in any of the antisense treated grafts placed in cholesterol fed animals. In contrast to the lesions seen in all sections of six week control grafts, only rare, isolated macrophages were observed in areas of minimal neointimal accumulation in antisense treated vessels, while their hypertrophied medial layers remained free of any macrophage infiltration as did the walls of the adjacent carotid arteries. This example, shows that an intraoperative transfection of vein segments with these antisense ODN can inhibit the long term development of neointima in vein grafts. Furthermore, this example shows that by blocking neointimal hyperplasia during the initial post-operative period, when the graft is responding both to the acute injury of surgery as well as the chronic hemodynamic stresses of the arterial environment, grafts can be genetically engineered to adapt to arterial stresses via medial hypertrophy. Plaque formation and the accumulation of lipid laden macrophages occur within 4 to 6 weeks in the vein graft walls of cholesterol fed rabbits. The "arterialized" grafts of this example not only displayed a structural, biochemical and mechanical similarity to native arteries, but also proved resistant to this accelerated vein graft atherosclerosis.

Despite the elimination of significant neointimal hyperplasia and its predilection for atherosclerosis in antisense ODN transfected grafts, these vessels

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5 retained the capacity for adaptation to the arterial environment. The reduction of wall stress to normal arterial levels is known to be an important aspect of neointima formation in vein grafts and this example shows that the medial hypertrophy of antisense treated grafts succeeded in reducing the luminal radius/vessel thickness ratio, which is proportional to wall stress, to a level comparable to those of the carotid arteries into which the grafts were placed. This example demonstrates, for the first time, the efficacy of an intraoperative gene therapy approach in altering vein graft adaptation *ex-vivo*, to produce a markedly improved graft that is resistant to failure.

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WHAT IS CLAIMED IS:

1. A method for inhibiting vascular cellular activity of cells associated with vascular lesion formation in mammals, said method comprising:
administering to the site associated with vascular lesion formation an effective
dosage of at least one antisense oligomer sequence to at least one gene expressing a
cyclin or a cyclin dependent kinase wherein the antisense sequence inhibits the
expression of the gene.
2. A method according to claim 1 wherein the cyclin is cyclin A, B1, B2, C, D1, D2, D3, E or cyclin X(p46) and the cyclin dependent kinase is cdc2, cdk2, cdk4, or cdk5.
3. A method according to claim 1 wherein at least two antisense sequences each complementary to a nucleic acid different cyclin or cyclin dependent kinase are administered to the site of vascular lesion formation.
4. A method according to claim 1 wherein the vascular lesion is associated with cardiovascular angioplasty and the antisense sequence is applied to the angioplasty site to reduce restenosis.
5. A method according to claim 1 wherein the antisense sequence to cyclin or cyclin dependent kinase is administered in combination with an antisense sequence against proliferating cell nuclear antigen (PCNA).
6. A method according to claim 1 wherein the antisense sequence is administered in a liposome.
7. A method according to claim 6 wherein the liposome contains hemagglutinating virus of Japan (HVJ).
8. A method according to claim 1 wherein the antisense sequence is administered intraluminally, intramurally, or periadventitiously.
9. A method according to claim 5 wherein the antisense sequences are against cdc2 and PCNA.

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10. A method according to claim 3 wherein the antisense sequences are against cyclin A and cdc2.

11. A method according to claim 3 wherein the antisense sequences are against cyclin B and cdc2.

12. A method according to claim 3 wherein the antisense sequences are against cyclin A and cdk2.

13. A method according to claim 1 wherein said oligomer comprises an alkyl- or aryl-phosphate oligomer, a phosphotriester oligomer, a carbamate oligomer, a sulfamate oligomer, a morpholino oligomer, a phosphorothioate oligomer, a phosphorodithioate oligomer, a formacetal oligomer, a thioformacetal oligomer or a peptide oligomer.

14. A method according to claim 13 wherein the antisense oligomer is 12 to 30 nucleotides in length and is stabilized by one or more phosphorothioate linkages.

15. The method of claim 1 wherein the oligomer is administered in a composite formulated with (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride as a lipofectin.

16. A method according to claim 6, wherein said liposome is a cationic, neutral, or anionic liposome.

17. A method for treating vein grafts comprising contacting a vein graft *ex-vivo* with an antisense oligomer sequence to at least one gene expressing a cyclin or a cyclin dependent kinase wherein the antisense sequence inhibits the expression of the gene.

18. A method according to claim 17 wherein the cyclin is cyclin A, B1, B2, C, D1, D2, D3, E or cyclin X(p46) and the cyclin dependent kinase is cdc2, cdk2, cdk4, or cdk5.

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19. A method according to claim 17 wherein the antisense sequence to cyclin or cyclin dependent kinase is administered in combination with an antisense sequence against proliferating cell nuclear antigen (PCNA).
20. A method according to claim 17 wherein the antisense sequence is administered in a liposome.
21. A method according to claim 20 wherein the liposome contains hemagglutinating virus of Japan (HVJ).
22. A method according to claim 20, wherein said liposome is a cationic, neutral, or anionic liposome.
23. A method according to claim 19 wherein the antisense sequences are against cdc2 and PCNA.
24. A method according to claim 19 wherein the antisense sequences are against cyclin A and cdc2.
25. A method according to claim 19 wherein the antisense sequences are against cyclin B and cdc2.
26. A method according to claim 19 wherein the antisense sequences are against cyclin A and cdk2.
27. A method according to claim 17 wherein said oligomer comprises an alkyl- or aryl-phosphate oligomer, a phosphotriester oligomer, a carbamate oligomer, a sulfamate oligomer, a morpholino oligomer, a phosphorothioate oligomer, a phosphorodithioate oligomer, a formacetal oligomer, a thioformacetal oligomer or a peptide oligomer.
28. A method according to claim 27 wherein the antisense oligomer is 12 to 30 nucleotides in length and is stabilized by one or more phosphorothioate linkages.

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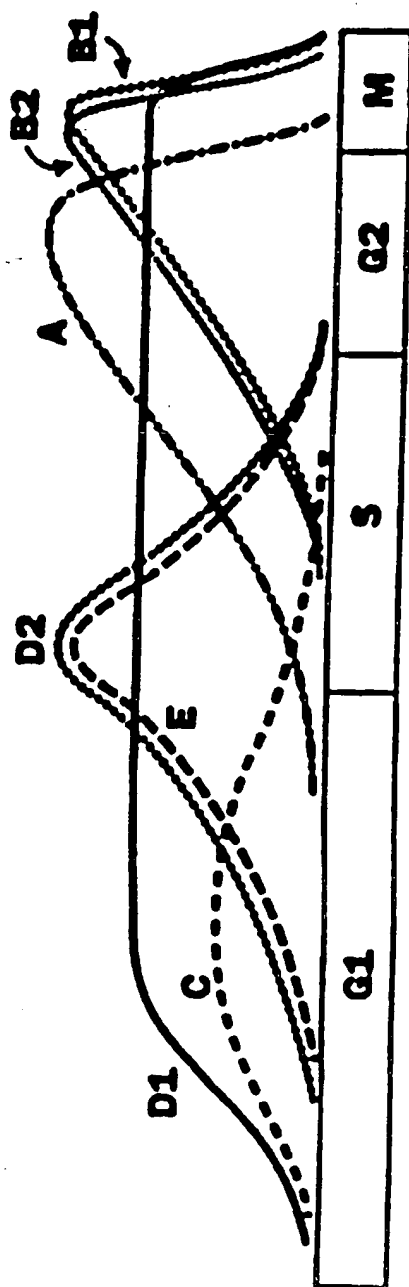


FIGURE 1

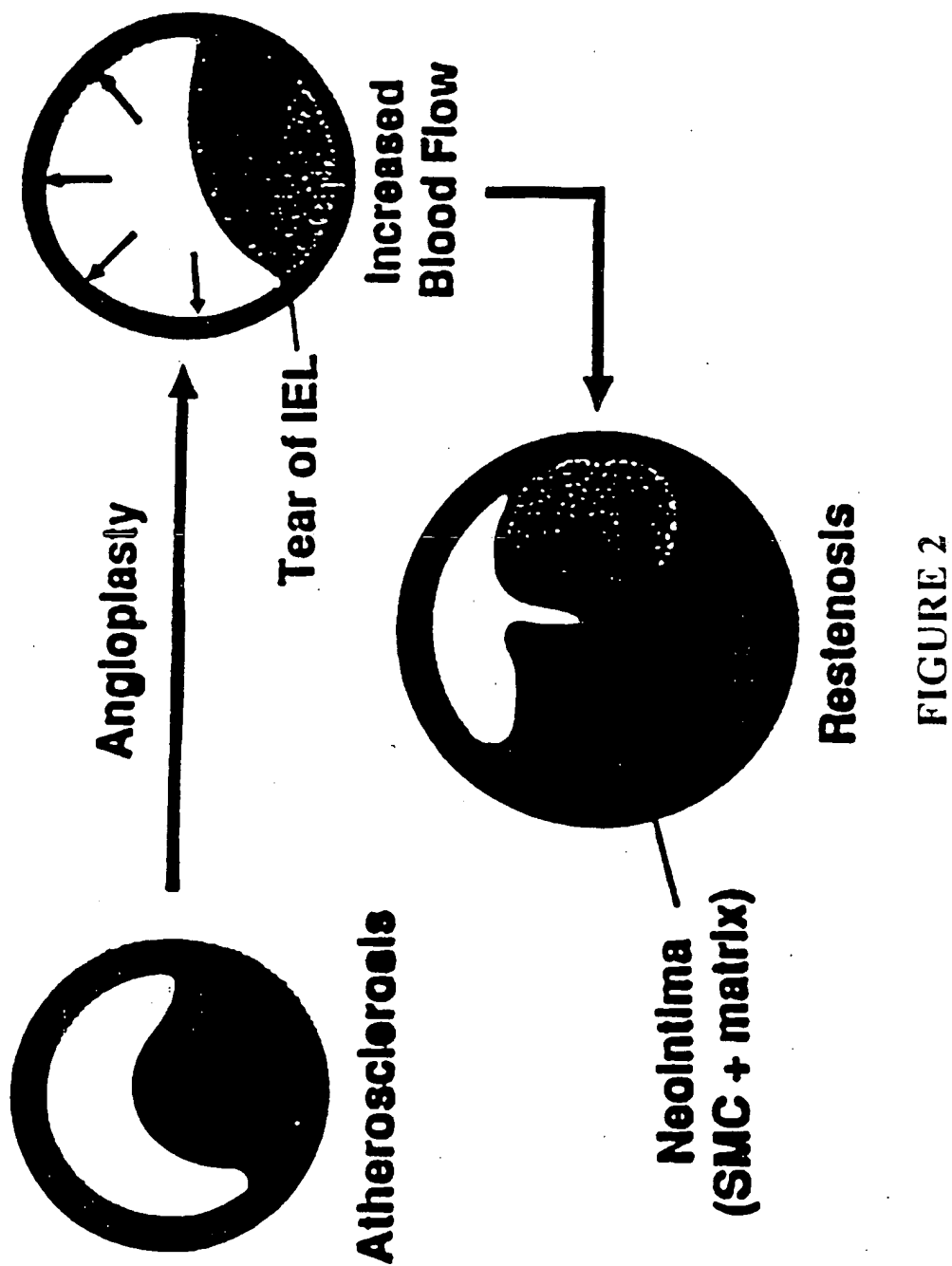


FIGURE 2

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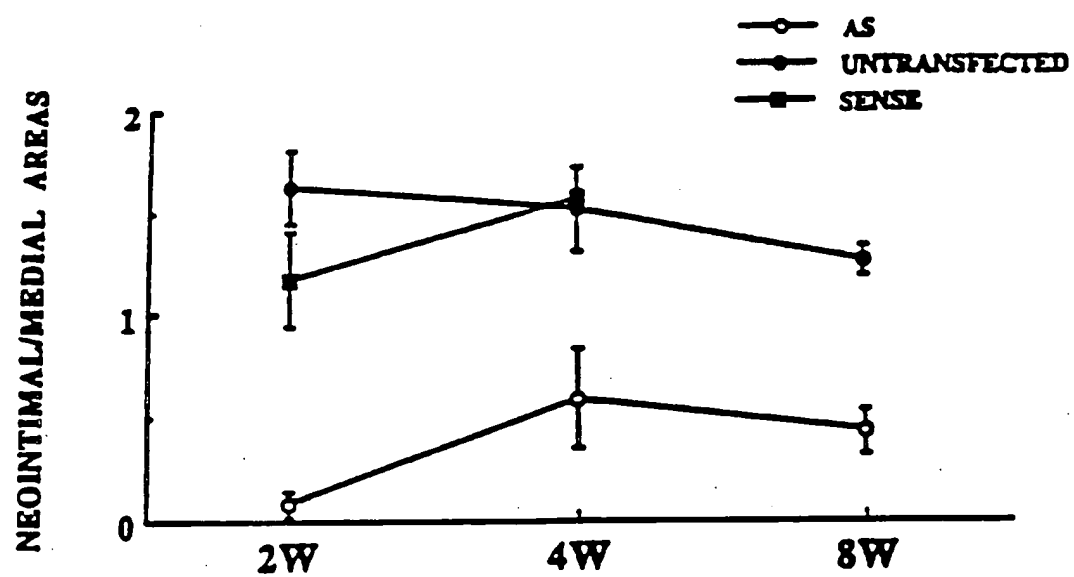


FIGURE 3

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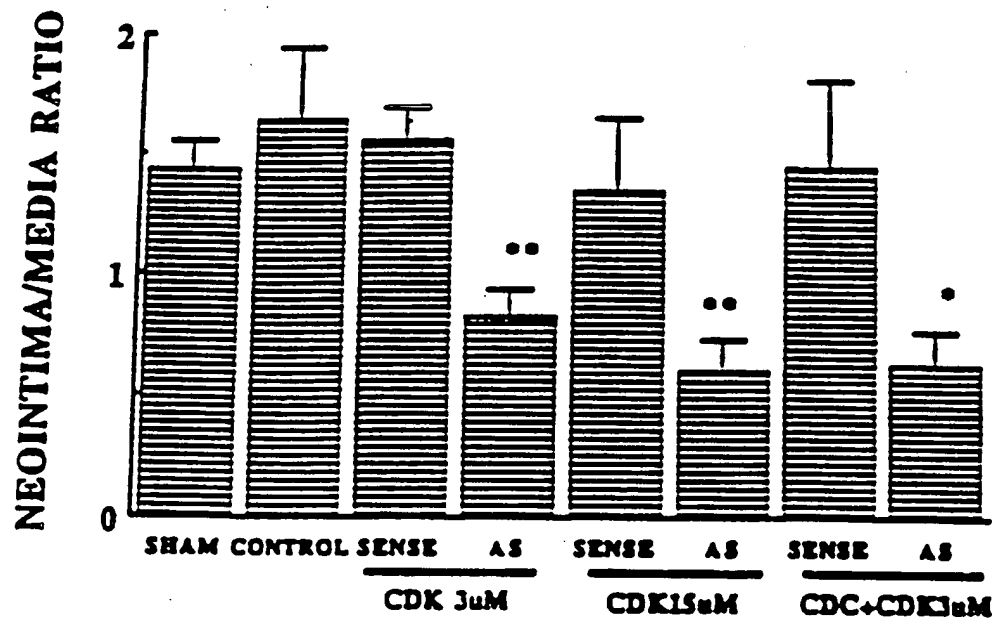


FIGURE 4

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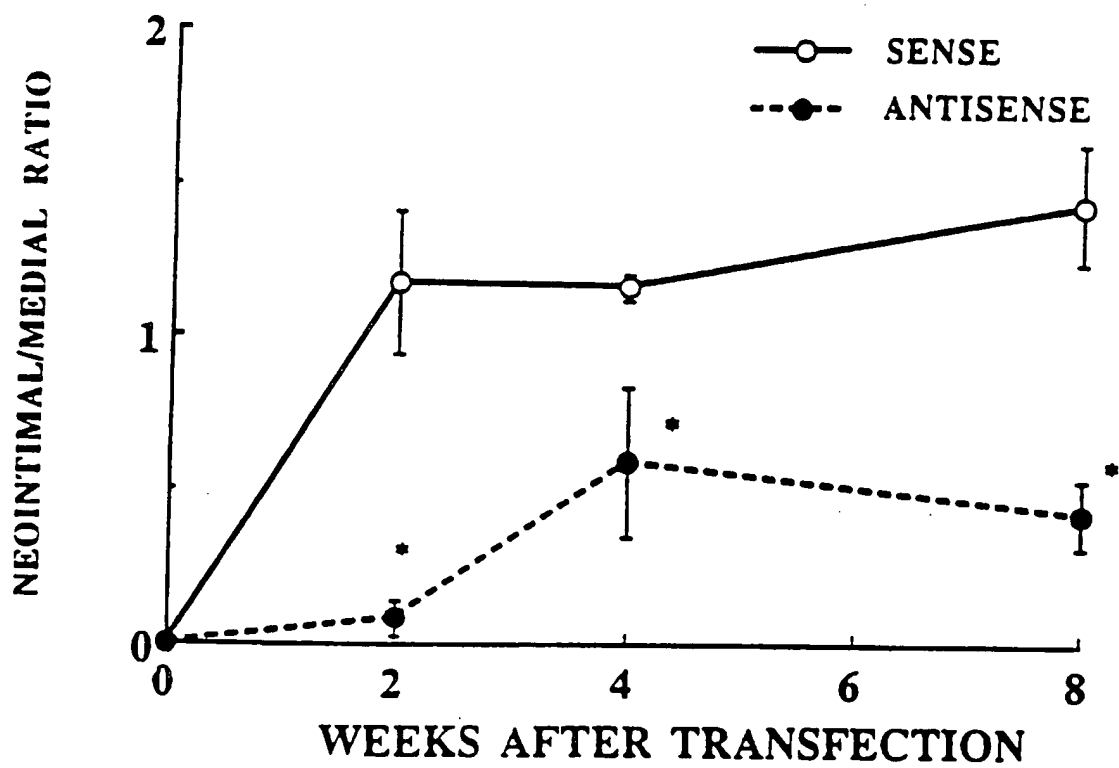


FIGURE 5

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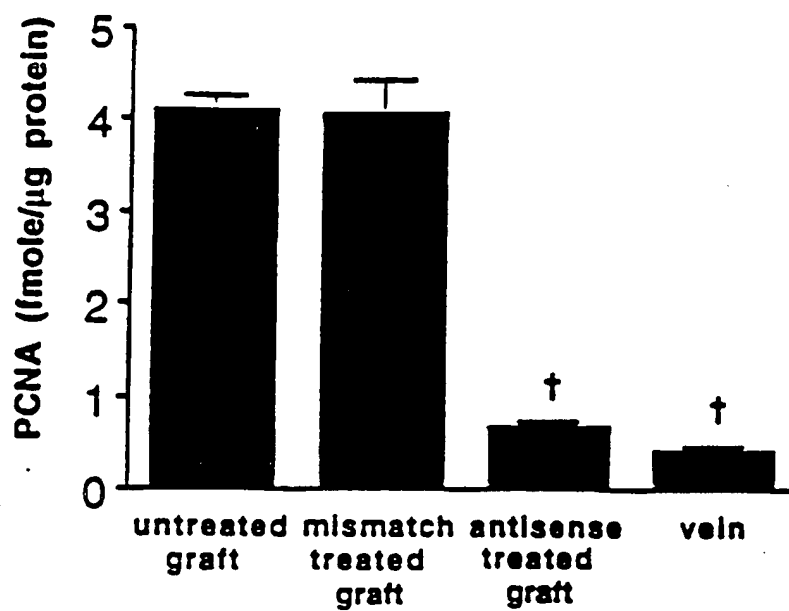


FIGURE 6A

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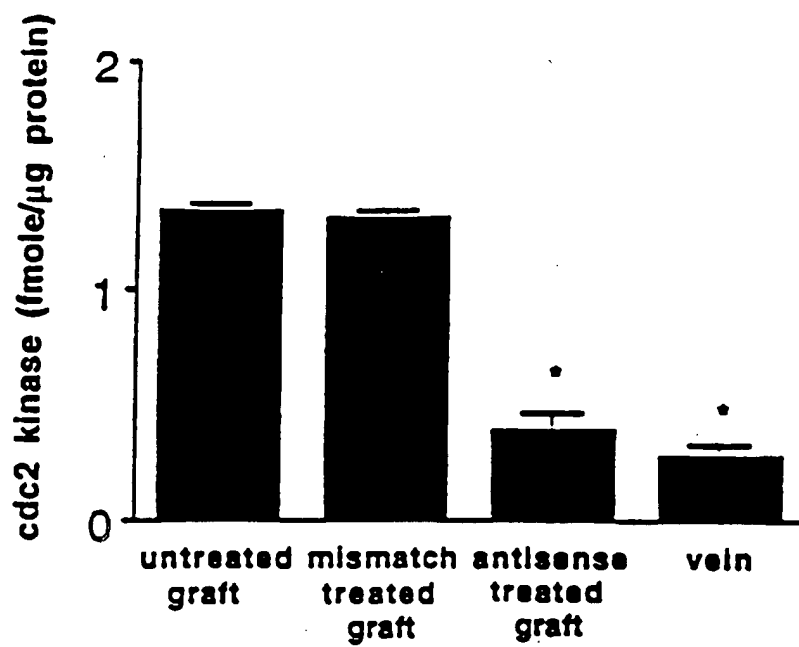


FIGURE 6B

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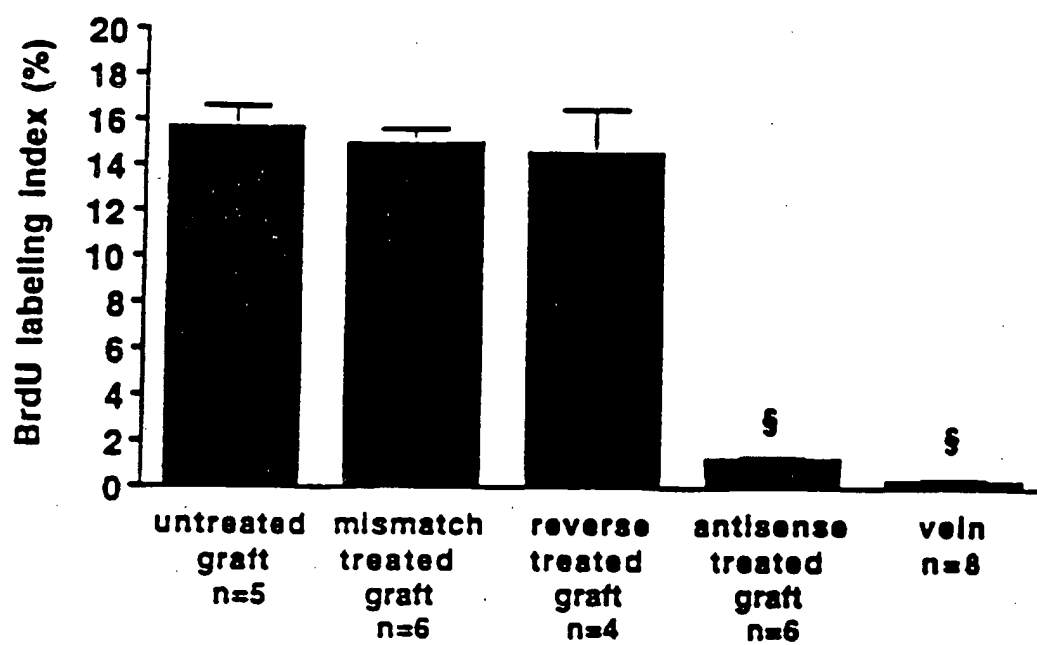


FIGURE 6C

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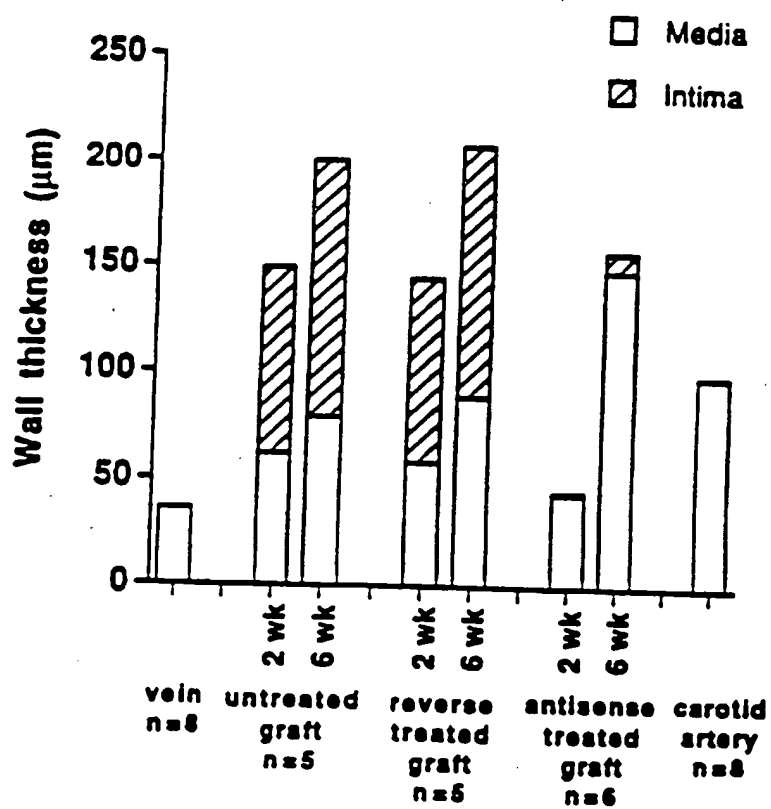


FIGURE 7A

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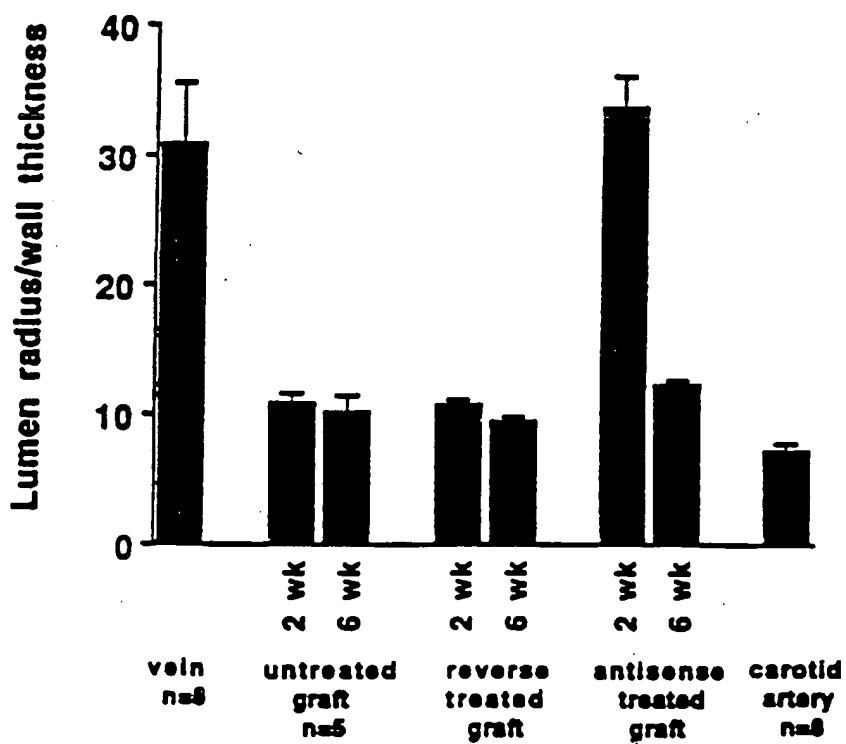


FIGURE 7B

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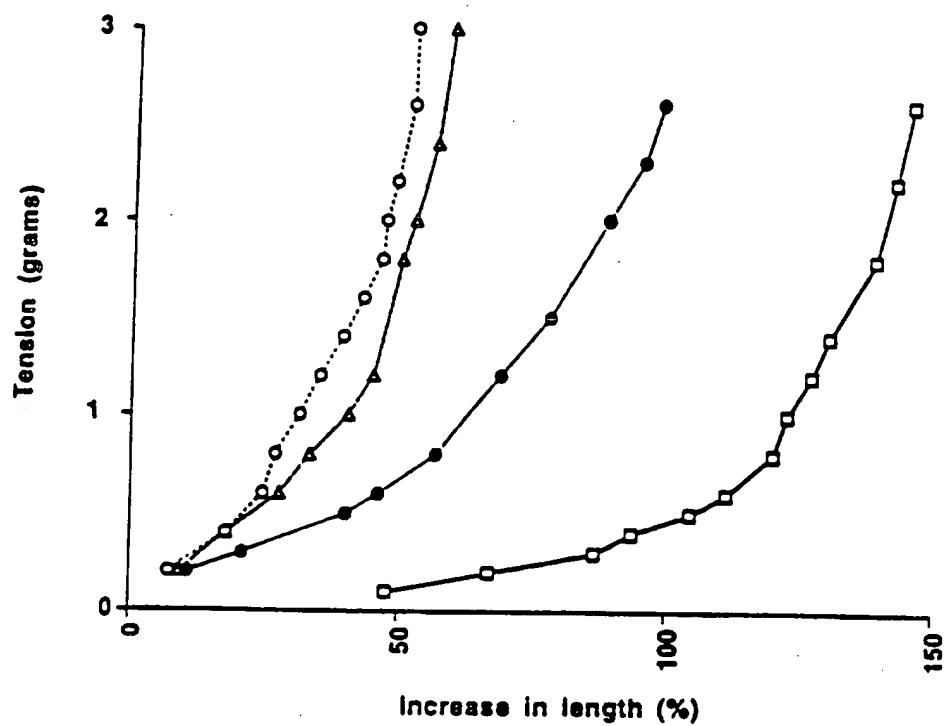


FIGURE 8

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/11, A61K 31/70, 9/127, C07H 21/00, C12N 15/88	A3	(11) International Publication Number: WO 96/25491 (43) International Publication Date: 22 August 1996 (22.08.96)
(21) International Application Number: PCT/US96/02064 (22) International Filing Date: 15 February 1996 (15.02.96) (30) Priority Data: 08/389,926 16 February 1995 (16.02.95) US (71) Applicant: THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY [US/US]; Office of Technology Licensing, Suite 350, 900 Welch Road, Palo Alto, CA 94304-1850 (US). (72) Inventor: DZAU, Victor, J.; 12101 Dawn Lane, Los Altos Hills, CA 94022 (US). (74) Agent: HUGHES, A., Blair, Banner & Allegretti, Ltd., Ten South Wacker Drive, Chicago, IL 60606 (US).	(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the International search report: 17 October 1996 (17.10.96)	
(54) Title: INHIBITION OF PROLIFERATION OF VASCULAR SMOOTH MUSCLE CELL (57) Abstract This invention encompasses a method for inhibiting vascular cellular activity of cells associated with vascular lesion formation in mammals which involves administering an effective dosage of at least one antisense sequence to at least one gene expressing a cyclin or a cyclin dependent kinase which inhibits the expression of the gene. More particularly, the invention involves administering antisense sequences which inhibit the expression of cyclin A, B1, B2, C, D1, D2, D3, E or cyclin X (p46) cyclin X and cyclin dependent kinase cdc2, cdk2, cdk4 or cdk5. It is preferable to use two antisense sequences each from a different cyclin or cyclin dependent kinase. The cyclin or cyclin kinase depending kinase dosage is preferably administered in combination with proliferating cell nuclear antigen (PCNA). Antisense methods and compositions directed to inhibiting the expression of growth factors such as TGF- β ₁ , TGF, bFGF, PDGF are also contemplated by the present invention. The antisense sequences are incorporated into liposomes, particularly liposomes containing HVJ and which are directly administered intraluminally, intramurally or periadventitiously. The methods of this invention are useful in treating a broad spectrum of vascular lesions such as lesions in the carotid femoral and renal arteries, particularly lesions resulting from renal dialysis fistulas. The invention is particularly useful in treating vascular grafts <i>ex-vivo</i> and prior to surgical grafting.		

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INTERNATIONAL SEARCH REPORT

International Application No

PC1/US 96/02064

A. CLASSIFICATION F SUBJECT MATTER

IPC 6 C12N15/11 A61K31/70 A61K9/127 C07H21/00 C12N15/88

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07H A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 26888 (UNIV LELAND STANFORD JUNIOR) 24 November 1994	1-16
Y	see the whole document	17-28

O,X	CIRCULATION 90 (4 PART 2). 189, October 1994, XP002011371	17-20, 22,23
	MANN, M. ET AL.: "Genetically engineered vein grafts: Resistant to neointima formation and adapted to the arterial circulation."	
Y	see abstract 0474	17-28
	& 67TH SCIENTIFIC SESSIONS OF THE AMERICAN HEART ASSOCIATION, DALLAS, TEXAS, USA, NOVEMBER 14-17, 1994. ,	

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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

22 August 1996

Date of mailing of the international search report

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Authorized officer

Andres, S

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/02064

C.(Continuation) D. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, September 1993, WASHINGTON US, pages 8474-8478, XP002011372 MORISHITA, R. ET AL.: "Single intraluminal delivery of antisense cdc2 kinase and proliferating-cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia" cited in the application see the whole document</p> <p>---</p>	1-9, 13-16
X	<p>THE JOURNAL OF CLINICAL INVESTIGATION, vol. 93, no. 4, April 1994, pages 1458-1464, XP002011373 MORISHITA, R. ET AL.: "Intimal hyperplasia after vascular injury is inhibited by antisense cdk2 kinase oligonucleotides" see the whole document</p> <p>---</p>	1-4,6-8, 13-16
X	<p>CLINICAL RESEARCH, vol. 41, no. 2, April 1993, page 211A XP002011374 MORISHITA, R. ET AL.: "Novel intraluminal molecular delivery of antisense cdc2 kinase and cyclin A oligonucleotides inhibits neointimal hyperplasia" see abstract</p> <p>---</p>	1-4,6-8, 10,16
Y		5,9, 13-16
Y	<p>CIRCULATION 86 ;538-47, August 1992, XP002011375 SPEIR, E. & EPSTEIN, S.: "Inhibition of smooth muscle cell proliferation by an antisense oligodeoxynucleotide targeting the messenger RNA encoding proliferating cell nuclear antigen" cited in the application see the whole document</p> <p>---</p>	5,9
Y	<p>MOLECULAR PHARMACOLOGY, vol. 41, no. 6, June 1992, pages 1023-1033, XP002011376 BENNETT, F. ET AL.: "Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides" see abstract</p> <p>---</p>	13-16

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INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CURRENT OPINION IN CARDIOLOGY, (1994 NOV) 9 (6) 641-9, XP000579719 BRYAN, A. ET AL.: "The biology of saphenous vein graft occlusion: etiology and strategies for prevention." see page 647, left-hand column, line 1 - right-hand column, line 13 ---	17-28
P,X	PROC. NATL. ACAD. SCI. U. S. A. (1995), 92(10), 4502-6 , May 1995, XP002011377 MANN, M. ET AL.: "Genetic engineering of vein grafts resistant to atherosclerosis" see the whole document ---	17-23
P,X	SURGICAL FORUM 46 (0). 258-260, 22 October 1995, XP000579717 MANN, M. ET AL.: "Genetic engineering of vein grafts resistant to failure: Mechanism of anti-atherogenic effect." see the whole document -----	17-19,23

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/02064

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US96/02064

FURTHER INFORMATION CONTINUED FR M PCT/ISA/210

Remark: Although claims 1-16 (as far as in vivo methods are concerned) are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

information on patent family members

PC1/US 96/02064

Form PCT/ISA/210 (patent family annex) (July 1992)